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13. ABSTRACT *(Maximum 200 Words)*

The effect of bone morphogenetic protein-2 (BMP-2) on breast cancer cell growth was not known at the time of our study. During past three years we systematically investigated the role of BMP-2 on human breast cancer cell growth and the underlying mechanism. The aim was to identify novel therapeutic application for BMP-2 in breast cancer cell growth inhibition. We showed that BMP-2 inhibits the growth of both estradiol-sensitive (MCF-7) and insensitive (MDA MB 231) human breast cancer cells. Next we demonstrated that BMP-2 inhibits estradiol-induced growth of both MCF-7 and MDA MB 231 cells by inhibiting mitogen activated protein kinase (MAPK) activity. In addition BMP-2 also induced phosphorylation of retinoblastoma protein (pRb). Phosphorylated pRb is a potent inhibitor for cell cycle progression. Thus we show that BMP-2 intercepts cell signaling for growth of cancer cells by two very powerful mechanisms. Since our goal was to use BMP-2 therapeutically, we showed that adenovirus-directed BMP-2 expression inhibits breast tumor growth in an animal model. In addition, we wanted to study the role of BMP-2 on bone metastasis of breast cancer cells, since one of the biggest problems associated with breast cancer is its secondary localization to bone. Currently we are designing experiments towards this goal and are testing them as a long term extension of this project.

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INTRODUCTION:

At the time we started to investigate the effect of bone morphogenetic protein-2 (BMP-2) on breast cancer cell growth, it was not known if and how BMP-2 will act on these cells. During past three years we systematically investigated the role of BMP-2 on human breast cancer cell growth and the underlying mechanism. The aim was to identify novel therapeutic application for BMP-2 in breast cancer cell growth inhibition. First we show that BMP-2 inhibits the growth of both estradiol-sensitive (MCF-7) and insensitive (MDA MB 231) human breast cancer cells [1, 2], thus setting the stage for our study to unravel the underlying mechanism for this effect. Next we demonstrated that BMP-2 inhibits estradiol-induced growth of both MCF-7 and MDA MB 231 cells in culture, by inhibiting mitogen activated protein kinase (MAPK) activity. In addition BMP-2 also increased expression of p21, a protein that inhibits cell cycle progression. This effect was translated in inhibiting BMP-2-induced cyclin dependent kinase (cdk) activity and finally phosphorylation of retinoblastoma protein (pRb). Phosphorylated pRb is a potent inhibitor for cell cycle progression. Thus we show that BMP-2 intercepts cell signaling for growth of cancer cells by two very powerful mechanisms. Since our goal was to use BMP-2 therapeutically, we next attempted to study the effect of BMP-2 on breast cancer cell growth in an animal model. In addition, we wanted to study the role of BMP-2 on bone metastasis of breast cancer cells, since one of the biggest problems associated with breast cancer is its secondary localization to bone, Thus we designed experiments towards this goal and tested them in the fianl year of this project.

BODY:

Task 1. **To correlate the levels of BMP-2 and BMP-2 (BMPR) expression in breast cancer cell lines and tissue samples with their bone metastasis status (months 1-12)**

- **Perform Northern as well as the RT-PCR analysis of BMP-2 and BMPR expression in different ER negative and ER positive cells and in different breast tumor tissue samples (months 1-6).**

- **Analyze the expression level of BMP-2 and BMPR different ER negative and ER positive cells and in different breast tumor tissue samples (months 6-7).**

We have analyzed for the expression levels of BMP-2 in MDA MB 231 and MCF-7 cells by Western blotting technique and a pool of available breast cancer cell lines for expression of BMPR by RTPCR analysis. We found that the cells express abundant levels of BMP-2 but the expression of receptors in breast cancer cells are extremely low (data shown in previous reports). Since the cancer tumor repository was moved from San

Antonio to Houston I was unable to access the breast tumor samples to examine their BMPR level.

- **Correlate the metastatic potential of the breast cancer cells using the animal model described above *by in vivo* study (months 6-12).**

- **Correlate metastatic property of the tissue samples with the expression levels of BMP-2 and BMPR in these samples (months 7-8).**

We have described the preliminary results for developing an *in vivo* model for bone metastasis in last years report. We had used MDA MB 231 and MCF7 cells in that experiment and provided X-ray analysis to show osteolysis in both the cases. Following that we have acquired an adenovirus expressing BMP-2 (Ad BMP-2) and had used the fund provided by this grant to generate a high titer stock of this virus from Quantum Biotechnologies, Montreal, Canada. In one experiment we have infected MDA MB 231 cells with 150 moi of Ad BMP-2 and have injected these cells 20h post infection in mammary fat pads of female nude mice. In the control group of mice we used MDA MB 231 cells infected with empty adenoviral vector for injection. We have determined the tumor growth for 20 days and have found that Ad BMP-2 infected cells formed significantly small tumors than those in the control group (Fig.1). This result confirms our *in vitro* observation and suggests that BMP-2 can inhibit breast tumor growth. This result needs to be confirmed first of all by analyzing BMP-2 expression in the tumors at the end experiment to ensure that the effect observed was indeed due to the difference in level of BMP-2 in the control and experimental group. These experiments are currently in progress. We are also doing similar studies with MCF7 cells to find out if the growth of tumors from these cells can also be inhibited by BMP-2 expression in an animal model.

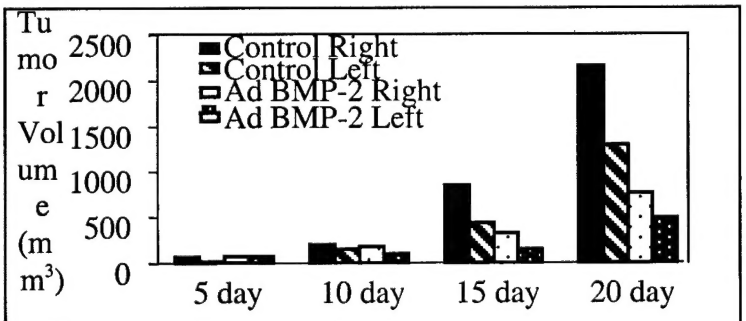

Fig 1. Inhibition of Tumor Growth in nude mice injected with MDA MB 231 cells infected with Ad BMP-2. 5 x 10^6 cells were infected with Ad BMP-2 at a moi of 150 and were injected at 20h post infection into mammary fad pads of 4 week old female nude mice (n=5). Cells infected with control adenovirus vector were injected in the control group of mice (n=5). Tumor volumes were noted at 5, 10, 15 and 20days of injection.

Since we have shown earlier that bone osteolysis was detected in this xenograft model using both MDA MB 231 and MCF7 cell injection in mammary fat pads of nude mice, we are currently analyzing the bone from mice injected with AD BMP-2 infected MDA MB 231 cells to determine the effect of BMP-2 in bone metastasis.

Task 2. **To prove the clinical importance of BMP-2 expression and bone metastasis of breast cancer cells by genetically engineering the BMP-2 status of breast cancer cells and study the metastatic phenotype of the altered cells using an *in vivo* model of metastasis (months 6-24).**

- **Genetical alteration of the BMP-2 protein and receptor status (in cells chosen based on the results of task 1) by using stable transfection of corresponding cDNAs. These experiments will be initiated simultaneously with some of the experiments described in the Task 1. This period will be necessary to establish all the clonal cell lines and analyze their BMP protein and receptor expression profiles and finally to test the chemotactic properties of these cells. As the cell lines will be available, they will be tested for their metastatic potential by the *in vivo* assay in the animal model described above (months 6 - 24).**

We have shown last year in our annual report that the MDA MB 231 and MCF7 cells and many other human breast cancer cells express very low levels of BMPR. To make the cells more responsive to BMP-2 we designed an alternate approach. BMP-2 signals through 2 types of membrane bound receptors, type I and type II. Both of these receptors can bind BMP-2 and activates type I receptor. There are two type I BMPR, IA and IB. We decided to overexpress contitutively active forms of BMPRIA and BMPRIB in MDA MB 231 cells. These forms of BMPR can constitutively transduce BMP-2-mediated signal even in absence of BMP-2. Thus the cells expressing these receptors will show BMP-2 effect more effectively. With this experiment in mind we have acquired adenovirus expressing these BMPRs in constitutive active forms. We have expanded the viral stock in our lab to determine the efficiency and the expression kinetics of these virus-based receptor proteins. These proteins expressed by these viral vectors are tagged with hemagglutinin (HA) for easier detection. We had infected MDA MB 231 cells with these viruses individually and had determined the expression of HA tagged BMPRs by Western Blotting using anti-HA antibody. In this experiment we could

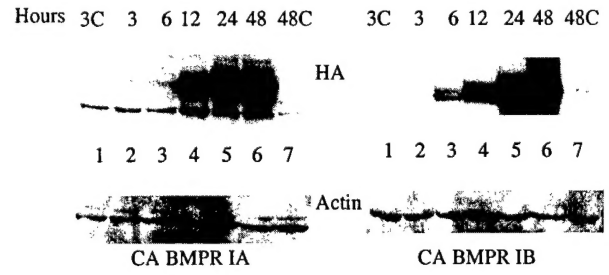

Fig 2. Expression kinetics of hemaglutinin (HA)-tagged CA BMPR IA and IB following infection of MDA MB 231 cells with the respective adenoviral expression vectors. Cells were infected at a moi of 150 and were harvested at 3, 6, 12, 24 and 48h post infection. Lysates were analyzed by Western Blotting using anti- (HA) antibody. 3C and 48C indicates lysates from cell lines infected with control adenoviral vectors. Bottom panels show immunoblot of the same membrane using anti-actin antibody for loading control.

detect HA expression as early as 6h post infection and the expression remained sustained for 48h (Fig.2). We are planning to make a high titre stock of these viral vectors for a systematic *in vitro* study to show that expression of these proteins in human breast cancer cells can also inhibit cell growth. In the long run we plan to investigate the efficacy of this approach in reducing breast tumor growth and bone metastasis in an animal model.

We will perform similar experiments with MCF 7 for the aims described in Task 4.

Task 3. **To study differential effect of BMP-2 on ER-positive and ER-negative breast cancer cell growth and to investigate the underlying mechanism (months 6-18).**

- **Study effect of BMP-2 on ER-negative breast cancer cells *in vitro* by Flowcytometric analysis of cell cycle progression (months 6-8).**

Study the underlying mechanism of BMP-2 induced growth regulation of breast cancer cells. This part will include determination of the effect of BMP-2 on the MAPK pathway by enzymatic activity assay for MAPK, studying growth kinetics of breast cancer cells in the presence of MEK inhibitor and identification of other targets for BMP-2 action in these cells (months 9-24).

We completed this task. The results are given in our annual reports of 2000 and 2001 and are detailed in two publications (Appendix 1 and 2) [1, 2]. The results showed that BMP-2 can inhibit the growth of both ER positive and ER-negative breast cancer cells by inhibiting MAPK activity.

Task 4. **To correlate the growth inhibitory effect of BMP-2 on ER-positive breast cancer cells *in vivo* (months 18-36).**

- **To study autocrine BMP-2 action (*in vitro*): Analysis of effect of estradiol on growth kinetics of MCF-7 cells stably transfected with BMP-2 cDNA (months 18-24).**

Since we did not find any significant reduction in BMP-2 expression in MCF 7 cells and detected change in BMPR levels, we decided to approach this task by using constitutively active BMPRs, as described in details in task 2.

- **To study autocrine BMP-2 action (*in vivo*): Xenograft tumor formation assay of MCF-7 cells stably transfected with BMP-2 cDNA or vector alone (months 24-36).**

We will perform experiments for this task using MCF7 cells expressing constitutively active forms of BMPR type IA and IB (described in task 2 in details).

Key Research Accomplishments:

- Identification of BMP-2 as a potential *in vitro* growth inhibitor for the breast cancer cells irrespective of their estrogen responsiveness.
- Understanding of the underlying mechanism of BMP-2-induced inhibition of breast cancer cell growth *in vitro*.
- Establishment of *in vivo* mouse model for studying breast cancer cell growth and skeletal metastasis.
- Confirming *in vitro* growth inhibitory properties of BMP-2 in inhibiting breast tumor growth in an animal model.

Reportable Outcome:

- **Abstract:** BMP-2 inhibits EGF-induced MDA-MB231 breast cancer cell growth via increased expression of p21 WAF1 cyclin kinase inhibitor (CKI) by posttranscriptional mechanism. N. Ghosh-Choudhury, S.L. Abboud and G. Ghosh-Choudhury. American Society of Bone and Mineral Research 21st Annual meeting, 1999. **(Poster Presentation)**
- **Abstract:** Mechanism for inhibitory role of BMP-2 on growth of estrogen-responsive and non-responsive human breast cancer cells. N. Ghosh-Choudhury, S.L. Abboud and G. Ghosh-Choudhury. International Conference Bone Morphogenetic Proteins 2000. **(Oral Presentation)**
- **Abstract: Ghosh-Choudhury N,** Abboud SL, Ghosh-Choudhury G. Phosphatidylinositol 3 kinase (PI 3-K) and Akt serine threonine kinase regulate BMP-2 induced osteoblast differentiation and smad-dependent transcription of BMP-2 gene. J Bone Miner Res 16: S144, 2001 **(Oral Presentation)**
- **Abstract: Ghosh-Choudhury N,** Woodruff K, Tang YP, Abboud SL, Ghosh-Choudhury G. Positive and negative regulation of osteoblast and MCF-7 human breast cancer cell growth in response to BMP-2: Involvement of mitogen activated protein kinase (MAPK).93rd Annual Meeting ; American Association for Cancer Research; 2002.

- **Manuscripts:**

1. ***Ghosh-Choudhury N**, Woodruff K, Qi1 W, Celeste A, Abboud SL and Ghosh-Choudhury G, Bone Morphogenetic Protein-2 (BMP-2) Blocks MDA MB 231 Human Breast Cancer Cell Proliferation by Inhibiting Cyclin-Dependent Kinase-Mediated Retinoblastoma Protein (pRb) Phosphorylation. Bichem Biophys Res Comm., 272: 705-711, 2000.

2. ***Ghosh-Choudhury, N**, Ghosh-Choudhury G, Celeste A, Ghosh P M, Moyer M, Abboud SL and Kreisberg J, Bone Morphogenetic Protein-2 induces cyclin kinase

inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol treated MCF-7 human breast cancer cells. Biochimica et Biophysics Acta., 1497:186-196, 2000.

3. ***Ghosh-Choudhury N**, Ghosh Choudhury G, Harris MA, Wozney J, Mundy GR, Abboud SL, Harris SE. Autoregulation of mouse BMP-2 gene transcription is directed by the proximal promoter unit. Biochem Biophys Res Comm. 286:101-108, 2001.

4. Ghosh-Choudhury G, Zang J-H, **Ghosh-Choudhury N,** Abboud HE. Ceramide blocks PDGF-induced DNA synthesis in mesangial cells via inhibition of Akt kinase in the absence of apoptosis. Biochem Biophys Res Comm 286: 1183-1190, 2001.

Conclusions:

We have analyzed the role of a novel protein in the breast cancer cell growth mechanism. This protein, bone morphogenetic protein-2 (BMP-2), has been characterized before as a modulator of bone cell growth. We show that BMP-2 can significantly inhibit the growth of breast cancer cells *in vitro*. We have studied this growth inhibitory role of BMP-2 in estrogen-responsive and non-responsive breast cancer cells and have found that it inhibits the growth of both the cell types with equal potency. In estradiol-responsive human breast cancer cells, BMP-2 can inhibit estradiol-induced growth of these cells. We have also identified the mechanism by which BMP-2 inhibits the growth of these cells ([1]). We also showed that BMP-2 inhibit MDA MB 231 cell growth by increasing p21 inhibitor of cyclin dependent kinases (cdk). This increase in p21 results in increase in growth inhibitory phosphorylated form of retinoblastoma protein [2].

We are developed an *in vi*vo model animal to test this *in vitro* phenomenon. We injected MDA MB 231 cells infected with an adenoviral vector expressing BMP-2 (Ad BMP-2) into mammary fat pads of female nude mice. The cells developed into tumors, the volume of which were significantly smaller than those obtained with cells infected with adenovirus containing control empty vector (Fig.1). The results, though preliminary, show that BMP-2 is effective *in vivo* to inhibit breast tumor growth. This is the first demonstration of BMP-2-mediated reduction of breast tumor size in an animal model. W e are now designing experiments to confirm these results using constitutively active forms of BMP receptors. We are also designing experiments to study the effect of BMP-2 on bone metastasis of breast tumors. Since secondary tumors are more devastating in breast cancer patients, if BMP-2 shows inhibitory effect on bone metastasis of breast cancer cells it will prove to be a clinically important molecule for the breast cancer patients.

Reference:

1. Ghosh-Choudhury, N., G. Ghosh-Choudhury, A. Celeste, P.M. Ghosh, M. Moyer, S.L. Abboud, and J. Kreisberg, *Bone morphogenetic protein-2 induces cyclin kinase*

inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF- 7 human breast cancer cells. Biochim Biophys Acta, 2000. **1497**(2): p. 186-96.
2. Ghosh-Choudhury, N., K. Woodruff, W. Qi, A. Celeste, S.L. Abboud, and G. Ghosh Choudhury, *Bone morphogenetic protein-2 blocks MDA MB 231 human breast cancer cell proliferation by inhibiting cyclin-dependent kinase-mediated retinoblastoma protein phosphorylation.* Biochem Biophys Res Commun, 2000. **272**(3): p. 705-11.

APPENDIX

Reprints:

1) ***Ghosh-Choudhury N**, Woodruff K, Qi1 W, Celeste A, Abboud SL and Ghosh-Choudhury G, Bone Morphogenetic Protein-2 (BMP-2) Blocks MDA MB 231 Human Breast Cancer Cell Proliferation by Inhibiting Cyclin-Dependent Kinase-Mediated Retinoblastoma Protein (pRb) Phosphorylation. Bichem Biophys Res Comm., 272: 705-711, 2000.

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Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells

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Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells

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Abstract

The biologic effects and mechanisms by which bone morphogenetic proteins (BMPs) function in breast cancer cells are not well defined. A member of this family of growth and differentiation factors, BMP-2, inhibited both basal and estradiol-induced growth of MCF-7 breast tumor cells in culture. Flow cytometric analysis showed that in the presence of BMP-2, 62% and 45% of estradiol-stimulated MCF-7 cells progressed to S-phase at 24 h and 48 h, respectively. Estradiol mediates growth of human breast cancer cells by stimulating cyclins and cyclin-dependent kinases (CDKs). BMP-2 significantly increased the level of the cyclin kinase inhibitor, p21, which in turn associated with and inactivated cyclin D1. BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity. Also estradiol-induced CDK2 activity was inhibited by BMP-2. This inhibition of CDK activity resulted in hypophosphorylation of retinoblastoma protein thus keeping it in its active form. These data provide the first evidence by which BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells.

Keywords: BMP-2; pRb; p21; Breast cancer cell

1. Introduction

17-β-Estradiol (estradiol) acts as a potent mitogen for breast epithelial cells and thus causes increased cell growth, both in vivo and in vitro. In estradiol-responsive human breast cancer cells like MCF-7, the hormone responsiveness is mediated by estrogen receptors (ERs) [1]. Activation of ER stimulates cyclin-dependent kinases (CDKs) to induce proliferation of MCF-7 cells. On the other hand, increased expression of cyclin kinase inhibitor p21 blocks CDK activity necessary for retinoblastoma protein (pRb) phosphorylation [2]. These results indicate that a concerted effect of different cell cycle proteins regulates cell cycle progression.

Bone morphogenetic proteins (BMPs), BMP 1–9, constitute a group of growth factors that are in-

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volved in ectopic bone formation [3]. They are produced as pro-mature forms, which are processed to active dimers of the mature region in a manner similar to the transforming growth factor β (TGFβ) [4]. Due to this similarity, BMPs are categorized as members of the TGFβ super family. In addition to other functions during embryonic development and limb formation, BMPs regulate chondrogenesis and osteogenesis [5–7]. Though BMPs have been detected in osteosarcomas and soft tissue carcinomas, the role of BMPs in breast cancer is still unclear. Like TGFβ receptors, multiple BMP receptors have recently been identified. They form two closely related groups known as type I and type II receptors, which contain multiple members. Both the receptor types have serine/threonine kinase activity in their cytoplasmic domains [8,9]. Three downstream target molecules for BMP-2 have recently been identified. These targets are Smad 1, Smad 5 and Smad 8. BMP-2 stimulates association of Smad 1 with the BMP receptor followed by phosphorylation of the Smad 1 C-terminus by the type I receptor [10,11]. Receptor-phosphorylated Smad 1 undergoes heterodimerization with the tumor suppressor protein Smad 4. This heterodimer then translocates to the nucleus and participates in transcription of genes [10,12].

Recently, Nakaoka et al. demonstrated that BMP-2 inhibits smooth muscle cell proliferation [13]. BMP-2 blocks serum and androgen-induced growth of human prostate cancer cells in culture [14]. We have recently shown that BMP-2 at a moderate dose blocked PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells without any effect on matrix gene expression [15,16]. Also a high dose of recombinant BMP-2 has recently been shown to inhibit soft agar growth of a variety of tumor samples including breast tumor [17]. However, the mechanism of BMP-2-mediated inhibition of tumor cell growth is not known. In this report, we demonstrate the inhibitory effect of BMP-2 on estradiol-induced MCF-7 human breast cancer cell proliferation in culture. BMP-2 increases the levels of cyclin kinase inhibitor p21 without any effect on estradiol-induced cyclin D1 expression. We also show that BMP-2 inhibits estradiol-induced cyclin D1-associated kinase and CDK2 activity with concomitant reduction of pRb phosphorylation. This is the first elucidation of the signaling mechanisms, involved in BMP-2-mediated inhibition of estradiol-induced breast cancer cell growth.

2. Materials and methods

Tissue culture materials were purchased from Gibco. Estradiol, phenyl methyl sulphonyl fluoride (PMSF), soybean inhibitor, leupeptin, myelin basic protein, propidium iodide and RNase A were obtained from Sigma. Histone H1 was purchased from Boehringer Mannheim. GST-pRb was obtained from Santa Cruz. Micro BCA reagent and enhanced chemiluminescence (ECL) kit were purchased from Pierce. Protein A-Sepharose CL 4B was purchased from Pharmacia. All antibodies were obtained from Santa Cruz. Recombinant BMP-2 was obtained from Genetics Institute.

MCF-7 breast cancer cells were obtained from Dr. Robert Klebe (Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio) and were routinely maintained in DMEM–F12 medium containing 10% new born calf serum. These MCF-7 cells are highly responsive to estradiol and tamoxifen. For experiments designed to test the mitogenic effect of estradiol, cells were grown in complete medium for 48 h to reach subconfluency and then placed in phenol red-free and serum-free DMEM for 48 h before addition of estradiol. Treatment with serum-free medium slows the growth of cells because they tend to arrest at G0/G1 phase. For cell cycle analysis near confluent cells were used for serum-deprivation to arrest in G0/G1 phase before addition of estradiol to release them.

2.1. Flow cytometric analysis

MCF-7 cells were trypsinized and washed with phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol for 30 min at −20°C, centrifuged at 1500×*g* for 4 min, washed with PBS containing 1% bovine serum albumin (BSA) and resuspended in 150 μl PBS. For nuclear staining with propidium iodide, the cells were treated with 50 μl of 1 mg/ml RNase A (Sigma) followed by 100 μl of 100 μg/ml propidium iodide. The cells were incubated at 4°C for 18–24 h before they were analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry

Systems, San Jose, CA). Cells were illuminated with 200 mW of light at 488 nm produced by an argon-ion laser and the fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20 000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

2.2. MTT (3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) assay for cell proliferation

Proliferation of MCF-7 cells in response to estradiol was determined using the MTT assay as described elsewhere [18]. In brief, 50 μl of 5 mg/ml MTT was added to the culture medium of growing cells (1 ml medium/well) and incubated for 4 h at 37°C in a humidified atmosphere with 5% CO_2. The medium was removed and 200 μl of DMSO was added to each well. The absorbance of the dissolved dye was measured at 540 nm.

2.3. Immunoprecipitation and immunoblotting

Immunoprecipitation was carried out according to methods described elsewhere, with minor modifications [16,19]. In brief, cells were lysed in immunoprecipitation buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 50 mM NaCl, 0.1% Nonidet P-40, 50 μg/ml PMSF, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 1 μg/ml aprotinin) and cleared of cell debris by centrifugation at 4°C. Protein estimation was done in supernatant by a micro BCA assay. 200 μg protein was routinely precleared by incubating with 20 μl of swelled protein A-Sepharose beads for 1 h in the cold. The cleared supernatant was immunoprecipitated at 4°C for 18–24 h using 1 μg of antibody followed by addition of 20 μl swelled protein A-Sepharose beads for 1 h. The protein A-Sepharose beads containing the antigen–antibody complex were then gently washed three times with immunoprecipitation buffer before eluting the bound proteins in the sodium dodecyl sulfate (SDS)–polyacrylamide gel loading buffer.

Immunoblotting was performed essentially as previously described [20]. Briefly, the cleared cell lysates or immunoprecipitates were separated in SDS–polyacrylamide gels (12% or 7.5% depending on the protein sizes). The proteins were electrophoretically transferred onto Nytran membrane. Following the transfer, the proteins were incubated with blocking solution (50 mM Tris–HCl pH 7.4–150 mM NaCl–0.2% Tween 20 (TBST) containing 5% non-fat dry milk) for 1 h at room temperature, followed by overnight incubation in primary antibody solution prepared in TBST containing 1% BSA. The membrane was subsequently washed five times in TBST for 5 min each, before the horseradish peroxidase-conjugated secondary antibody was added in TBST for 1 h at room temperature. The membrane was finally washed in TBST, five times for 5 min each and the antigen–antibody complex was detected using an ECL kit (Pierce) as per manufacturer's recommendations.

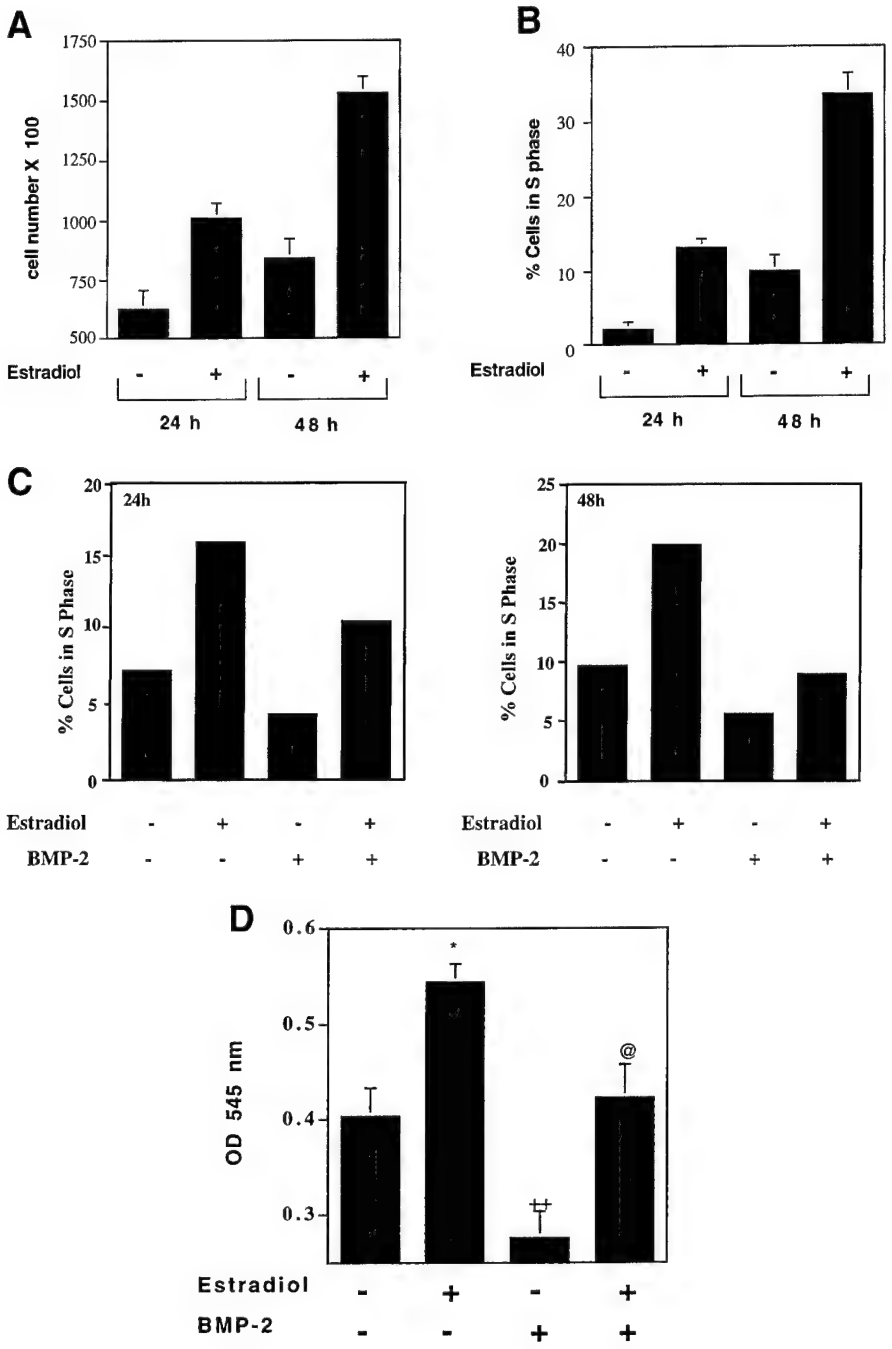
2.4. Cyclin D1-associated kinase and CDK2 assay

The assay was performed using the method of Gong et al. [21]. Briefly, cleared cell lysate was immunoprecipitated using antibody against cyclin D1 or CDK2 as described above. The immunecomplex beads were resuspended in kinase buffer (20 mM Tris–HCl pH 7.5 and 4 mM $MgCl_2$). To measure the cyclin D1-associated kinase activity, a fragment of pRb, that contains the in vivo phosphorylation sites, was used. For CDK2 activity, calf thymus histone H1 was used as a substrate. The reaction was carried out in the presence of 25 μM 'cold' ATP and 10 μCi [γ-^{32}P]ATP for 30 min at 37°C. The phosphorylated proteins were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography. Phosphorylation was quantitated using a densitometric scan of the phosphorylated bands in autoradiogram.

3. Results

3.1. Growth inhibition of MCF-7 cells by BMP-2

Estradiol is a potent mitogen for ER positive human MCF-7 breast carcinoma cells [22]. To establish the optimal conditions to assess the effect of BMP-2 on MCF-7 cell proliferation, MCF-7 cells were treated with 1 nM 17-β-estradiol for 24 and 48 h,



respectively, and the cell number was counted. As expected, estradiol increased the cell number at each time point (Fig. 1A). To examine the effect of estradiol on cell cycle progression, MCF-7 cells were subjected to flow cytometry. At 24 and 48 h, a significantly higher percentage of cells were in S-phase in the presence of estradiol, as compared to unstimulated control cells (Fig. 1B). At 48 h, 10% of cells entered the S-phase even in the absence of estradiol. This may be due to incomplete quiescence of MCF-7 breast tumor cells. An alternative possibility may be accumulation of mitogens in the culture medium during 48 h of incubation in the serum-deprived medium.

To determine the effect of BMP-2 on cell cycle progression of MCF-7 cells stimulated by estradiol,

Fig. 1. Effect of estradiol and BMP-2 on MCF-7 cell proliferation. (A) Confluent layers of MCF-7 cells in 35 mm tissue culture dishes were serum-starved for 48 h and incubated with 1 nM estradiol for 24 and 48 h. Cells in each dish were counted. Increase in cell number was 1.6- and 1.8-fold, respectively, at 24 and 48 h. (B) Flow cytometric analysis of estradiol-induced MCF-7 cell proliferation. Serum-deprived MCF-7 cells were grown in the presence or absence of 1 nM estradiol for 24 and 48 h. Cells were then trypsinized and analyzed by flow cytometry as described in Section 2. The percentage of cells in S-phase was plotted. The percentage of cells in S-phase was increased by 6.5- and 3.7-fold at 24 and 48 h, respectively. Means of triplicate determinations are shown in A and B. (C) Quantitation of flow cytometric analysis of cells treated with BMP-2 and estradiol. MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol for 24 h (left panel) and 48 h (right panel) before subjecting them to flow cytometric analysis. The percentage of cells in S-phase was plotted for each condition. (D) Effect of BMP-2 on estradiol-induced MCF-7 cell proliferation. 48 h serum-deprived MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol. MTT assay was performed as an index of cell proliferation as described in Section 2. Results are means ± S.E.M. of three independent experiments. $*P < 0.05$ vs. untreated cells. $++P < 0.05$ vs. untreated control. $@P < 0.05$ vs. estradiol-treated cells.
←

cells were incubated with estradiol for 24 and 48 h, either in presence or absence of BMP-2. BMP-2 inhibited estradiol-induced S-phase progression of these cells. Quantitation of these results shows that only 62% and 45% of estradiol-treated MCF-7 cells entered S-phase at 24 and 48 h, respectively, in the presence of BMP-2 (Fig. 1C, left and right panels). BMP-2 alone also inhibited S-phase entry of control cells, by 41% and 43% at 24 and 48 h, respectively. To determine if the effect of BMP-2 on estradiol-induced cell cycle progression correlated with cell growth, MTT assays were performed. Fig. 1D shows that BMP-2 significantly inhibited estradiol-stimulated as well as basal MCF-7 cell proliferation. A photomicrograph of MCF-7 cells in the absence and presence of BMP-2 is shown in Fig. 2. As evident, treatment of these cells for 48 h with BMP-2 does not have any toxic effect. Taken together, these results indicate that BMP-2 inhibits estradiol-induced cell growth by preventing the entry of MCF-7 cells into S-phase.

3.2. *BMP-2 stimulates expression of cyclin kinase inhibitor, p21, in estradiol-treated MCF-7 cells*

Progression of the cell cycle is regulated by a series of CDKs [23]. These serine/threonine kinases are positively regulated by cyclins [24,25]. One of the G1 phase cyclins, cyclin D1, is overexpressed in more than 50% of human breast adenocarcinomas [26–28]. We studied the effect of estradiol on cyclin D1 expression. In accordance with the previous report [29], estradiol treatment of MCF-7 cells increased the level of cyclin D1 (Fig. 3A, compare lane 2 with lane 1). However, pretreatment of MCF-7 cells with BMP-2 had no significant effect on estradiol-induced expression of cyclin D1 (Fig. 3A, compare lane 4 with lane 2). These data indicate that the effect of BMP-2 on estradiol-induced MCF-7 cell proliferation is not caused by the modulation of cyclin D1 levels during cell cycle progression.

CDK activity is also regulated by cyclin kinase inhibitors [25]. One such protein, p21, is a universal

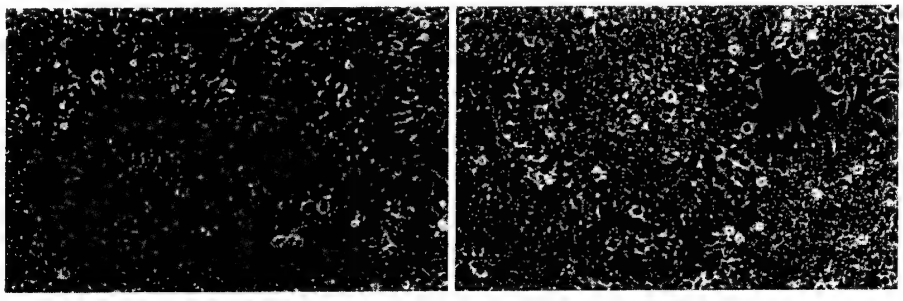


Fig. 2. Photomicrograph of MCF-7 cells in the presence and absence of BMP-2. Serum-deprived MCF-7 cells were incubated with BMP-2 for 48 h before taking the photograph. The phase contrast photomicrograph is shown.

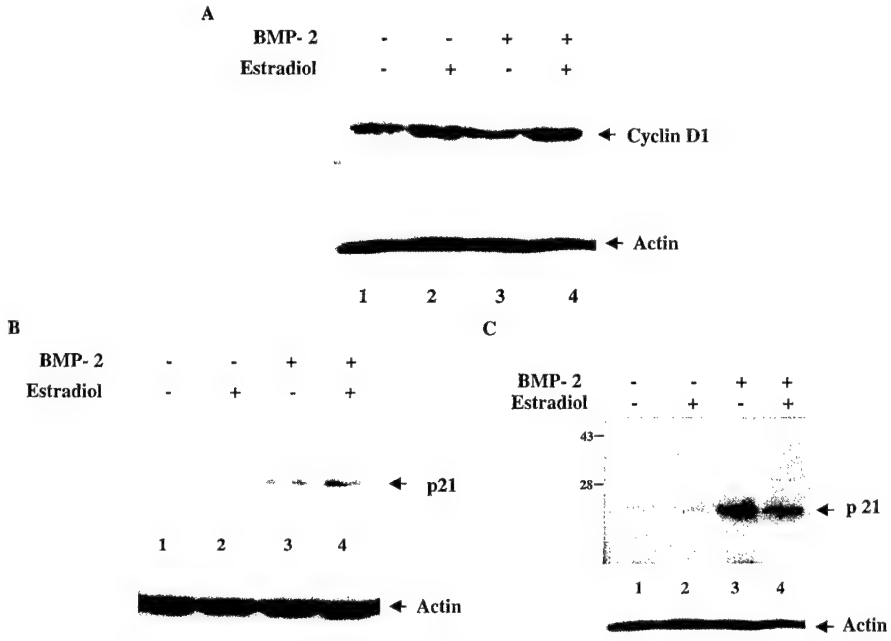


Fig. 3. (A) Effect of BMP-2 on estradiol-induced expression of cyclin D1 and p21. MCF-7 cells serum-deprived for 48 h were incubated with 1 nM estradiol in the presence and absence of 100 ng/ml BMP-2 for subsequent 48 h. Cleared cell lysates were analyzed by immunoblotting with cyclin D1. (B and C) Effect of BMP-2 on p21 expression. Serum-deprived MCF-7 cells were incubated with estradiol in the presence and absence of 100 ng/ml BMP-2 for 6 h (B) and 48 h (C), respectively. The lysates were immunoblotted with p21 antibody and the signal was developed by ECL. Lower panels show immunoblotting of the same lysates with anti-actin antibody to demonstrate equal loading of proteins in each lane.

inhibitor of CDKs, that interacts with multiple cyclin–CDK complexes. It thereby inhibits their kinase activity, which drives the cells through the cell cycle [2,30,31]. To understand the mechanism of BMP-2 inhibition of cell cycle progression, we studied its effect on p21 expression. MCF-7 cells were incubated with BMP-2 and estradiol for 6 and 48 h. At both time points, estradiol did not have any affect on p21 protein expression as determined by immunoblot analysis (Fig. 3B,C, compare lanes 2 with lanes 1 in both panels). In contrast, treatment of MCF-7 cells with BMP-2 alone significantly increased the level of p21 protein expression (Fig. 3B,C, compare lanes 3 with lanes 1). In the cells co-treated with BMP-2 and estradiol (lane 4), the level of p21 expression remained increased as compared to untreated and estradiol-treated cells. These data indicate that the inhibitory effect of BMP-2 on MCF-7 cell proliferation may partly be due to its effect on increased expression of p21.

3.3. *BMP-2 inhibits cyclin D1-associated kinase activity via p21*

For p21 to exert its inhibitory effect on cell cycle progression, it must associate with one of the cyclin–CDK complexes [24,25]. Since estradiol-induced increase in cyclin D1 levels resulted in cell progression, while BMP-2-induced increase in p21 levels caused G1 arrest, we analyzed the association of p21 with cyclin D1 under similar conditions. Lysates of serum-deprived MCF-7 cells treated with estradiol in the presence and absence of BMP-2 were immunoprecipitated with the antibody to p21, followed by immunoblotting with a cyclin D1 antibody. As shown in Fig. 4A, estradiol alone has no effect on association of cyclin D1 with p21. In contrast, p21 was found to be associated with cyclin D1 in cells treated with BMP-2 alone or in combination with estradiol (Fig. 4A, lanes 3 and 4). These data indicate that BMP-2 treatment causes an increased association of p21 with

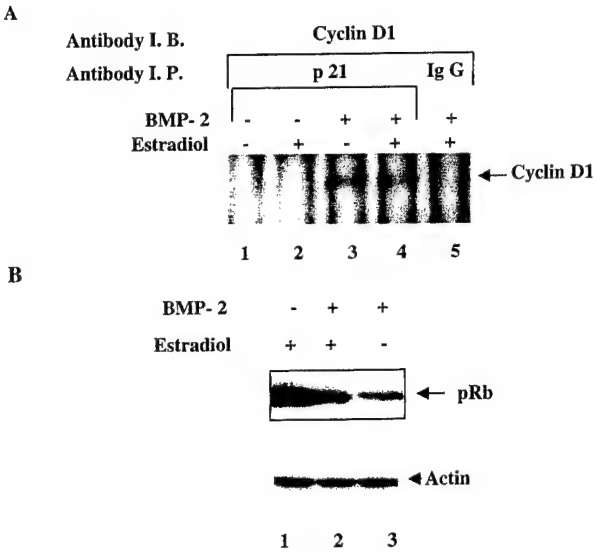


Fig. 4. (A) Effect of BMP-2 on association of p21 with cyclin D1. Cleared cell lysates from MCF-7 cells, treated as described in Fig. 3B,C, were immunoprecipitated (I.P.) with either anti-p21 or control IgG. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 12% SDS–polyacrylamide gel and were immunoblotted (I.B.) with anti-cyclin D1 antibody. Lanes 1–4 represent p21 immunoprecipitates. Lane 5 shows IgG immunoprecipitates. (B) Effect of BMP-2 on estradiol-induced cyclin D1-associated kinase activity. Serum-deprived MCF-7 cells were incubated with estradiol in the presence of BMP-2. The lysates were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for kinase activity in the presence of [γ-^{32}P]ATP using pRb as substrate. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows an immunoblot of same samples with actin antibody. →

cyclin D1 in MCF-7 cells which may result in the inhibition of cyclin D1-dependent kinase activity.

More recently, a role for p21 has been described as the assembly factor for cyclin D and CDK4/6 [32,33].

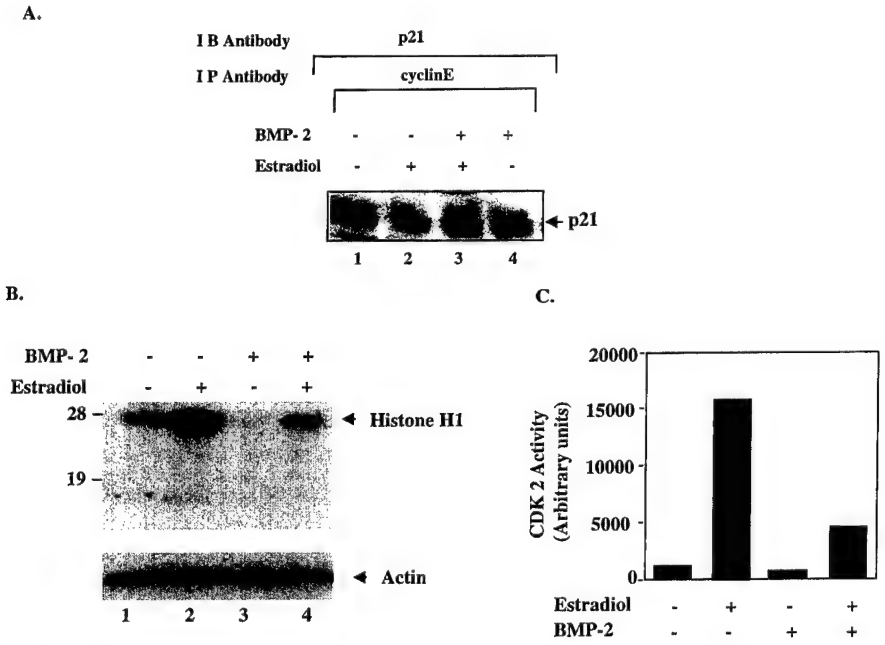


Fig. 5. (A) Effect of BMP-2 on association of p21 with cyclin E. Cleared cell lysates from MCF-7 cells, treated as described in Fig. 4, were immunoprecipitated (I.P.) with anti-cyclin E. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 15% SDS–polyacrylamide gel and were immunoblotted (I.B.) with anti-p21 antibody. Effect of BMP-2 on estradiol-induced CDK2 activity. (B) The cleared cell lysates from MCF-7 cells treated as described in Fig. 4 were immunoprecipitated with an anti-CDK2 antibody. The washed immunebeads were used in an in vitro immunecomplex kinase assay with histone H1 as substrate in the presence of [γ-^{32}P]ATP. The labeled proteins were separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows immunoblotting of the same lysates with anti-actin antibody. (C) Quantitation of histone H1 phosphorylation. The radioactivity incorporated into histone H1 in (A) was measured by a densitometric scan as described in Section 2 and plotted as histogram.

Another role of p21 in cell cycle is its inhibitory effect on CDK activity. Increased expression of p21 has been shown to inhibit both cyclin D1 and cyclin E-associated kinases [2]. To test this, lysates of MCF-7 cells treated with BMP-2 and estradiol were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for D1-associated kinase activity using pRb as in vitro substrate. As shown in Fig. 4B, BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity (compare lane 2 with lane 1). These data indicate that the inhibitory effect of BMP-2 may involve reduced pRb phosphorylation by cyclin D1-associated kinase (see below).

3.4. BMP-2 inhibits estradiol-induced CDK2 kinase activity and pRb phosphorylation

In the late G1 phase of cell cycle progression, E-type cyclin regulates CDK activity which is necessary for cells to enter and proceed through the S-phase [34]. p21 has been shown to regulate cyclin E via physical association. Since the p21 level was increased by BMP-2 (Fig. 3B,C), we tested if recombinant BMP-2 regulates p21 association with cyclin E. Cyclin E immunoprecipitates from lysates of estradiol or BMP-2 plus estradiol-treated MCF-7 cells were immunoblotted with p21 antibody. The results show that BMP-2 stimulated increased association of p21 with cyclin E in the presence and absence of estradiol as compared to estradiol alone (Fig. 5A, compare lanes 3 and 4 with lane 2). During late G1 and S-phase, cyclin E regulates CDK2 activity. To understand the mechanism of regulation of CDK2 in MCF-7 breast cancer cells, we analyzed the kinase activity associated with CDK2 in cells treated with estradiol in the presence or absence of BMP-2. Cell lysates were immunoprecipitated with a CDK2 antibody. The immunebeads were then used in an in vitro immunecomplex kinase assay with histone H1 as substrate in the presence of [γ-^{32}P]ATP. The data showed increased phosphorylation of histone H1 by CDK2 in cells treated with estradiol (Fig. 5B, compare lane 2 with 1). Estradiol-induced CDK2 activity was significantly inhibited by BMP-2 (Fig. 5B, compare lane 4 with lane 2). Quantitation of histone H1 phosphorylation showed 11-fold increase in CDK2 activity in the presence of estradiol (Fig. 5C), and BMP-2 inhibited 70% of estradiol-induced CDK2 activity (Fig. 5C). BMP-2 also partially inhibited the basal activity of CDK2 in MCF-7 cells. These data indicate that BMP-2-mediated inhibition of estradiol-induced MCF-7 proliferation may in part be due to its inhibitory effect on CDK2 activity.

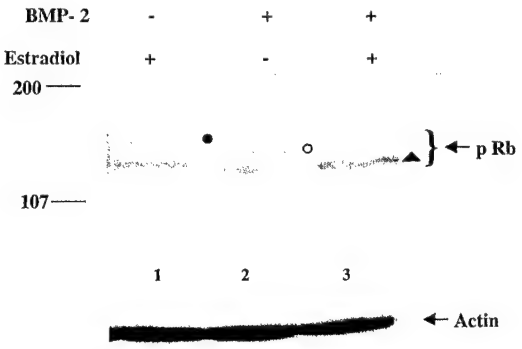


Fig. 6. Effect of BMP-2 on estradiol-induced pRb phosphorylation. The cleared cell lysates of MCF-7 cells, treated with estradiol in the presence and absence of BMP-2, were immunoblotted with an anti-pRb antibody. The migration of molecular weight markers (in kDa) is shown in the left margin. The filled circle shows hyperphosphorylated pRb in the highest phosphorylated form. The open circle shows pRb in intermediate phosphorylated form and the filled triangle shows pRb in hypophosphorylated form.

One of the targets of CDKs during cell cycle progression is pRb [24,35]. Hypophosphorylated pRb is active and inhibits cell cycle progression. Proliferative signals integrate into the nucleus to induce CDK-dependent phosphorylation of pRb rendering pRb inactive and resulting in DNA synthesis [35]. We showed in Fig. 4B that in in vitro kinase assay, pRb phosphorylation is reduced by BMP-2. To study the effect of BMP-2 on estradiol-induced pRb phosphorylation in MCF-7 cells, we analyzed lysates of MCF-7 cells by phosphorylation-dependent mobility shift assay. The degree of pRb phosphorylation is determined by its electrophoretic mobility, with hyperphosphorylated pRb forms migrating slower than the hypophosphorylated form in SDS gel. Lysates from MCF-7 cells, treated with estradiol or BMP-2 alone or with estradiol in the presence of BMP-2, were immunoblotted with an anti-pRb antibody. As shown in Fig. 6, estradiol caused hyperphosphorylation of pRb as indicated by the slower migration of this protein (lane 1, indicated by filled circle). Treatment of MCF-7 cells with BMP-2 alone resulted in the partial phosphorylation of pRb (lane 2, indicated

by open circle). In contrast, BMP-2 significantly inhibited hyperphosphorylation of pRb induced by estradiol and only hypophosphorylated pRb was the predominant form detected (Fig. 6, lane 3, indicated by filled triangle). These data indicate that the observed growth inhibitory effect of BMP-2 in estradiol-induced MCF-7 breast cancer cell proliferation could be caused by decreased CDK-dependent pRb phosphorylation.

4. Discussion

Our study demonstrates an inhibitory effect of BMP-2 on estradiol-induced MCF-7 breast cancer cell proliferation. BMP-2 stimulates increased expression of p21 cyclin kinase inhibitor. Consistent with this idea is our observation showing inhibition of estradiol-induced cyclin D1-associated kinase and CDK2 activity in response to BMP-2. Finally, we provide the first evidence that BMP-2 maintains the pRb tumor suppressor protein in a partially phosphorylated form.

Binding of estrogen to its receptor regulates a cohort of responsive genes that appears to regulate cell cycle progression. CDK4 and CDK6 form complexes with D-type cyclins during mid and late phases of G1, while CDK2 binds to cyclin E and D during late G1 [36–38]. One link between proliferative signals and cell cycle progression is provided by the induction of the secondary response genes, such as cyclin D1, following mitogenic stimulation [36]. In breast cancer, chromosome 11q13, which contains the cyclin D1 gene, has been shown to be amplified preferentially in ER positive tumors [39,40]. It has also been suggested that overexpression of cyclin D1 in MCF-7 cells causes them to proliferate in growth factor-deprived conditions [41]. In simvastatin or lovastatin-arrested MCF-7 human breast cancer cells, estrogen stimulates cell cycle entry by increasing cyclin D1 expression [42]. This effect of estrogen was due to transcriptional activation of the cyclin D1 gene by an estrogen-regulated response region present between the −944 bp of upstream sequences and the transcription start site of the cyclin D1 gene [42]. Cyclins, in association with CDKs and cyclin kinase inhibitors, control cell cycle progression through different phases of transitions and checkpoints. One of the cyclin kinase inhibitors, p21, has been shown to stimulate withdrawal from the cell cycle coupled to terminal differentiation [43]. Immunohistochemical analysis of breast carcinomas has shown that increased expression of p21 was associated with relapse-free survival [44]. p21 inhibits all the CDKs associated with cyclins A, D1 and E that are required for G1/S progression [31,45]. In addition to CDK inhibition, and thereby blocking cells from entering S-phase, p21 inhibits the DNA replication directly by binding to PCNA [46]. In the present study, we show that estradiol-induced S-phase entry of MCF-7 breast carcinoma cells is inhibited by the growth and differentiation factor BMP-2 (Fig. 1). Furthermore, our results demonstrate that BMP-2 causes increase in the levels of p21 protein as early as 6 h which sustains until 48 h (Fig. 3B,C). These data indicate that our observation of BMP-2-induced reduction in S-phase entry (Fig. 1C) and reduced proliferation (Fig. 1D) may be due to the increased expression of p21 protein (Fig. 3B,C). One of the mechanisms by which p21 blocks cells from entering S-phase is via interaction with cyclin D1 during G1 phase of the cell cycle, subsequently resulting in inhibition of CDK4 activity [2]. In the present study, we demonstrate association of p21 with cyclin D1 in the presence of BMP-2 (Fig. 4A). This may be the cause of reduced cyclin D1-associated kinase activity (Fig. 4B).

In addition to activation of cyclin D1/CDK4 during G1 phase, activation of cyclin E/CDK2 in late G1 is required for cells to progress through the cell cycle [38,47]. p21 inhibits both cyclin D1/CDK4 activity and cyclin E/CDK2 activity [2,30,48]. Treatment of MCF-7 breast cancer cells with estradiol stimulates cyclin D1-associated kinase (Fig. 4B) and CDK2 activity (Fig. 5B), which confirms the previous finding [47]. Pretreatment of cells with BMP-2, however, significantly blocked the estrogen-induced increase in both these kinase activities (Figs. 4B and 5B). Furthermore, BMP-2 increased the association of p21 with cyclin E (Fig. 5A). Our data for the first time demonstrate that BMP-2 targets the cell machinery at the level of CDKs. Thus one of the mechanisms by which BMP-2 inhibits MCF-7 cell proliferation is by inhibiting CDKs that are known to be activated in mid to late G1 and S-phases of cell cycle.

One of the targets of G1 CDKs is the tumor suppressor protein pRb [35]. This notion is established from various in vitro and in vivo studies. Cyclin D1/CDK4 complex can phosphorylate pRb in vitro [49]. The physiologic regulators that intercept CDK4/6 activity also block pRb phosphorylation. Similarly, overexpression of cyclin E in human osteosarcoma cells increases pRb phosphorylation [50]. pRb is also hyperphosphorylated in various breast cancer cells and tissues by cyclin E/CDK2 activity [51]. In tamoxifen-arrested MCF-7 cells, estradiol stimulates cyclin E/CDK2-dependent pRb phosphorylation [47]. We have also shown that treatment of serum-deprived MCF-7 cells with estradiol increased the level of hyperphosphorylated inactive pRb and that presence of BMP-2 during estradiol treatment caused reduction in the degree of pRb phosphorylation (Fig. 6). These observations describe one of the first mechanisms by which BMP-2 may inhibit MCF-7 breast cancer cell growth in culture.

In summary, we have demonstrated that BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells. This effect of BMP-2 appears to be mediated by inhibition of positive cell cycle regulatory proteins. Hyperproliferation of estrogen-responsive breast cancer cells is one of the major causes of tumor formation in early stages of breast cancer. Agents such as BMP-2 that inhibit estradiol-induced breast cancer cell proliferation may prove to be important therapeutic tools once their mechanisms of action are more thoroughly characterized.

Acknowledgements

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Ceramide Blocks PDGF-Induced DNA Synthesis in Mesangial Cells via Inhibition of Akt Kinase in the Absence of Apoptosis

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The mechanism of action of ceramide in glomerular mesangial cells has not been studied. We investigated the effect of C2 ceramide on the mitogenic signal transduction pathways induced by PDGF in mesangial cells. Increasing concentrations of C2 ceramide inhibited PDGF-induced DNA synthesis in a dose-dependent manner with maximum inhibition at 15 μM. This inhibition of DNA synthesis was associated with attenuation of PDGF-induced early response gene *c-fos* transcription. PDGF receptor β immunecomplex kinase assay showed no inhibitory effect of C2 ceramide on PDGF receptor tyrosine kinase activity. We have recently shown that the mitogenic effect of PDGF is mediated by the enzyme phosphatidylinositol (PI) 3 kinase in mesangial cells. C2 ceramide had no effect on PDGF-induced PDGFR-associated PI 3 kinase activity. These data indicate that inhibitory effect of C2 on PDGF-induced DNA synthesis is likely due to post-receptor and post-PI 3 kinase events. To address the mechanism of C2-mediated inhibition of DNA synthesis, we investigated the downstream target of PI 3 kinase, Akt. PDGF time-dependently increased Akt kinase activity in a PI 3 kinase-dependent manner. Incubation of mesangial cells with C2 ceramide inhibited PDGF-induced Akt activity. Akt kinase inhibits apoptosis of cells via phosphorylation of multiple pro-apoptotic proteins. However, inhibition of Akt activity by C2 ceramide did not induce apoptosis in mesangial cells. These data provide the first evidence that in mesangial cells, ceramide cross-talks with PI 3 kinase-dependent Akt kinase to inhibit PDGF-induced DNA synthesis without inducing apoptosis. © 2001 Academic Press

Key Words: **PDGF; PI 3 kinase; Akt kinase; ceramide; *c-fos;* apoptosis.**

Platelet-derived growth factor (PDGF) stimulates proliferation, migration, matrix expansion and secretion of growth factors and cytokines in mesangial cells vascular pericytes in the filtering units of the kidneys (1, 2). PDGF receptor β (PDGFR) possesses intrinsic tyrosine kinase activity (3). Binding of PDGF to the extracellular domain of PDGFR induces a conformational change, which stimulates dimerization of the receptor that relieves an inhibitory effect of the juxtamembrane domain resulting in trans phosphorylation of the receptor on specific tyrosine residues (4, 5). Tyrosine phosphorylation of PDGFR creates docking sites for SH-2 domain-containing signaling proteins such as phosphatidylinositol (PI) 3 kinase, RasGAP and SHP-2 among many (5, 6). Among these signaling proteins, binding of PI 3 kinase to the phosphorylated tyrosines 740 and 751 of the receptor is sufficient for PDGF-induced DNA synthesis in the PDGFR-transfected cells (7). We have recently shown that in mesangial cells, PI 3 kinase is necessary for PDGF-induced mesangial cell proliferation (8). However, the mechanism by which PI 3 kinase regulates proliferation of mesangial cells is not known.

One of the downstream targets of the PI 3 kinase is the serine threonine kinase Akt (also known as PKB), which is the cellular homolog of the transforming viral oncogene v-Akt (9, 10). Akt contains a pleckstrin homology domain in the N-terminus, a kinase domain with a threonine residue in the 308 position within the activation loop, and a regulatory serine phosphorylation site at residue 473 near the C-terminus (11). The PI 3 kinase products PI 3,4,5-trisphosphate and PI 3,4-bisphosphate bind to the PH domain of Akt to induce translocation of this kinase to the inner leaflet of plasma membrane, where the constitutively active phosphoinositide-dependent kinase-1 (PDK1) phosphorylates threo-

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nine 308 in the activation loop of Akt (11, 12). A yet to be identified kinase named PDK2 then phosphorylates serine 473, resulting in full activation of this kinase. The most extensively studied biological activity of Akt is its role in opposing apoptosis (13). The substrates for Akt involved in cell death regulation include BAD, caspase 9 and the forkhead family transcription factors (14–16). Phosphorylation and inactivation of these proapoptotic proteins by Akt lead to inhibition of apoptosis and results in cell survival (13). However, the role of Akt in cell proliferation has not been studied.

The sphingolipid derivative ceramide elicits a variety of cellular function (17). Ceramide can induce differentiation of HL60 cells in response to vitamin D3 (18), TNFα and interferon-γ (19). Furthermore ceramide has recently emerged as a key regulator of cellular proliferation in certain target cells (17). However, this sphingolipid has been most extensively studied for its proapoptotic property. Thus production of ceramide by a variety of extracellular signals strongly correlates with subsequent cell death (20, 21). In accordance with this, cell permeable ceramide analogs, such as C2 ceramide, also induce cell death in many different cell types (22–26).

One of the downstream targets of ceramide is the ceramide activated protein phosphatase (CAPP), which is a member of the PP2A family of serine threonine phosphatases (27–29). Activation of the CAPP has been implicated in the dephosphorylation of Bcl-2, protein kinase Cα and *c-Jun* transcription factor (30–32). Also a mitochondrial PP2A, a target of ceramide, has been directly shown to dephosphorylate serine 70 of Bcl-2, and to result in the inhibition of its anti-apoptotic function (33). Additionally, ceramide has recently been shown to inhibit the Akt serine threonine kinase activity in the insulin signal transduction pathway, suggesting a role for this lipid in normal metabolic function (34). Inhibition of Akt activity by ceramide also disrupts the ability of Akt to prevent apoptosis by phosphorylating the proapoptotic substrates Bad, caspase 9 and the forkhead transcription factors (14–16). In the present study we used C2 ceramide as a potential inhibitor of Akt to examine the role of this kinase in apoptosis and PDGF-induced DNA synthesis in mesangial cells. We provide the first evidence that C2 ceramide blocked PDGF-induced DNA synthesis without affecting the PDGFR tyrosine kinase activity and PDGFR-associated PI 3 kinase activity in mesangial cells. C2 ceramide inhibited PDGF-induced Akt serine threonine kinase activity with concomitant attenuation of PDGF-induced *c-fos* gene transcription. Of interest is that C2-mediated inhibition of Akt kinase activity occurred without inducing apoptosis.

MATERIALS AND METHODS

Materials. Tissue culture reagents were purchased from Life Technology, Inc. Phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, Nonidet P-40 (NP40) and Hoechst 33258 were obtained from Sigma. Aprotinin was purchased from Bayer. Recombinant PDGF BB was purchased from R & D Systems. C2 ceramide was obtained from Calbiochem. All antibodies were purchased from UBI Inc. Dual luciferase assay kit was obtained from Promega Inc. [^{3}H]Thymidine and [γ-^{32}P]ATP were purchased from Amersham.

Cell culture. Glomerular mesangial cells from Sprague Dawley rat were grown in RPMI 1640 in the presence of 16% fetal bovine serum. To make the cells quiescent, they were grown to confluency and incubated in serum free RPMI 1640 for 24 h (35, 36). Serum-deprived cells were incubated with C2 ceramide for 1 h before stimulation with PDGF BB.

DNA synthesis. [^{3}H]Thymidine incorporation into trichloroacetic acid insoluble material was determined as a measure of DNA synthesis as described before (8, 35–37).

Immunoprecipitation and PDGFR tyrosine kinase assay. Cells were lysed in radio immunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 0.05% aprotinin and 1% NP-40) at 4°C for 30 min. The cells were scraped and centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was collected and protein content was measured using BioRad reagent. Equal amounts of protein were immunoprecipitated with a PDGFR-specific antibody and the tyrosine kinase activity of the receptor was measured using [γ-^{32}P]ATP as described (35, 37, 38). The radioactive proteins were separated by 7.5% SDS–polyacrylamide gel and the labeled proteins were visualized by autoradiography.

PI 3 kinase assay. PDGFR immunoprecipitates were resuspended in PI 3 kinase assay buffer (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl and 0.5 mM EGTA). 0.5 μl of 20 mg/ml PI in DMSO was added and the kinase activity was determined in the presence of 10 μCi [γ-^{32}P]ATP as described previously (8, 37, 39).

Akt kinase assay. Anti-Akt immunoprecipitates were resuspended in Akt kinase assay buffer (50 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM DTT, 1 mM Na_3VO_4 and 5 μM ATP). Akt immunecomplex kinase assay was performed using histone H2B as substrate in the presence of 10 μCi of [γ-^{32}P]ATP as described (40). Phosphorylated histone H2B was separated by 15% SDS–polyacrylamide gel and visualized by autoradiography.

Immunoblotting. Cell lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane and immunoblotted with Akt antibody as described before (35, 36, 40).

Transfection and luciferase assay. Mesangial cells were transfected with *c-fos* promoter-driven firefly luciferase reporter plasmid (Fos-LUC) using lipofectamine essentially as described (35, 36). A CMV Renilla luciferase plasmid was also cotransfected to determine the transfection efficiency. Luciferase activity was determined using dual luciferase assay kit according to the method provided by the vendor (35, 36). Transcriptional activity of *c-fos* promoter was expressed as a ratio of firefly and Renilla luciferase activity.

Hoechst staining for examining apoptotic cells. Serum-deprived mesangial cells in chamber slides were incubated with 15 μM C2 ceramide for 1 h followed by incubation with PDGF for 24 h. These conditions were identical to those used to measure PDGF-induced DNA synthesis. The cells were subsequently used for Hoechst staining as described (41). Briefly, the cells were carefully washed with PBS twice and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were washed with PBS twice and stained with 10 μg/ml of Hoechst 33258 for 30 min. The cells were washed again with PBS. As a positive control for apoptosis, mesangial cells

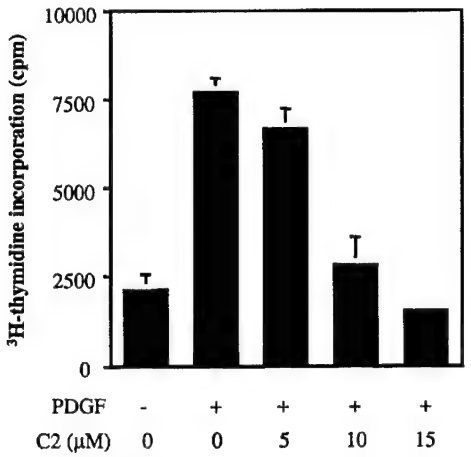

FIG. 1. Effect of C2 ceramide on PDGF-induced DNA synthesis in mesangial cells. Serum-deprived mesangial cells were incubated with different concentrations of C2 ceramide for 1 h before stimulation with 10 ng/ml PDGF BB for 24 h. During the last 4 h of incubation [^{3}H]thymidine was added. DNA synthesis was measured as the amount of [^{3}H]thymidine incorporated into TCA-insoluble precipitate (8, 35–37).

were treated with 200 μM H_2O_2 for 24 h. The cells in suspension were collected, fixed and stained in the Eppendorf tube and reseeded on a chamber slide before image analysis. The fluorescent images were observed and recorded with an Olympus IX70 microscope equipped with a mercury arch light source using an UplanApo FI 40× objective. The excitation and emission wavelengths were set at 360 and 460 nm, respectively. Signal collection and processing were achieved with Metaphore software.

RESULTS

Effect of C2 ceramide on PDGF-induced DNA synthesis. Ceramide elicits effects on many cell functions including proliferation, differentiation and apoptosis induced by different extracellular signals (17–23). We have previously shown that PDGF stimulates proliferation of mesangial cells (8). In the present study, we investigated the effect of ceramide on PDGF-induced DNA synthesis. Mesangial cells were incubated with different concentrations of C2 ceramide followed by treatment with PDGF. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis. C2 ceramide inhibited PDGF-induced DNA synthesis in a dose-dependent manner (Fig. 1). Maximal inhibition was obtained with a concentration of 15 μM (Fig. 1). These data indicate that ceramide regulates PDGF-induced mitogenic signaling in mesangial cells.

C2 ceramide does not regulate PDGF-induced PDGFR tyrosine kinase or PI 3 kinase activity. The most proximal biological effect of PDGF is the activation of its receptor tyrosine kinase activity, which is essential for all of its biological functions (4–6). We have previously shown that inhibition of PDGFR tyrosine kinase activity blocks PDGF-induced DNA synthesis in mesangial cells (42). Thus the inhibitory effect of C2 on PDGF-induced DNA synthesis (Fig. 1) may have resulted from inhibition of tyrosine kinase activity of PDGFR. Therefore we tested the effect of C2 ceramide on PDGFR tyrosine kinase activity. Mesangial cells were treated with C2 followed by incubation with PDGF. The cell lysates were immunoprecipitated with a PDGFR antibody and the receptor-associated tyrosine kinase activity was measured by immunecomplex kinase assay. PDGF stimulated PDGFR tyrosine kinase activity (Fig. 2, compare lane 2 with lane 1). C2 ceramide did not inhibit PDGF-induced intrinsic tyrosine kinase activity of the receptor (Fig. 2, compare lane 4 with lane 2). Note that C2 alone slightly stimulated the PDGFR tyrosine kinase activity.

We have recently shown that PI 3 kinase activity is necessary for PDGF-induced DNA synthesis in mesangial cells (8). Other investigators have shown that C2 ceramide can inhibit PI 3 kinase activity in a cell-specific manner (34, 43). Therefore the inhibitory effect of C2 on PDGF-induced DNA synthesis we observed (Fig. 1) may have been due to inhibition of PI 3 kinase activity. We therefore examined the effect of C2 on PI 3 kinase activity in mesangial cells. C2-treated cells were incubated with PDGF and PDGFR was immunoprecipitated from the cell lysate. PDGFR-associated PI 3 kinase activity was measured using PI as substrate. PDGF increased the PDGFR-associated PI 3 kinase activity (Fig. 3, compare lane 2 with lane 1). C2 did not have any significant effect on the PDGF-induced PI 3 kinase activity (Fig. 3, compare lane 4 with lane 2). These data indicate that C2 does not inhibit PI 3 kinase activity in mesangial cells. We conclude that the

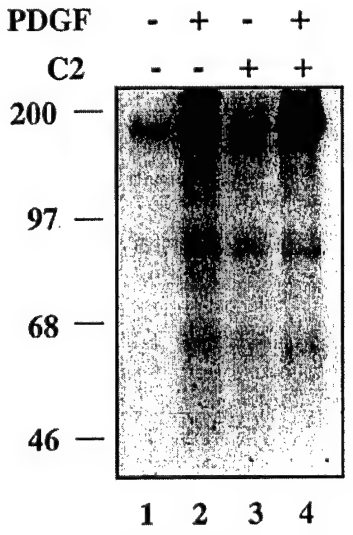

FIG. 2. Effect of C2 ceramide on PDGFR tyrosine kinase activity. Quiescent mesangial cells were incubated with 15 μM C2 for 1 h before stimulation with PDGF. Equal amounts of lysates were immunoprecipitated with PDGFR-specific antibody and the immunoprecipitates were used in an immunecomplex kinase as described under Materials and Methods. The numbers in the left margin indicate molecular mass markers in kilodaltons.

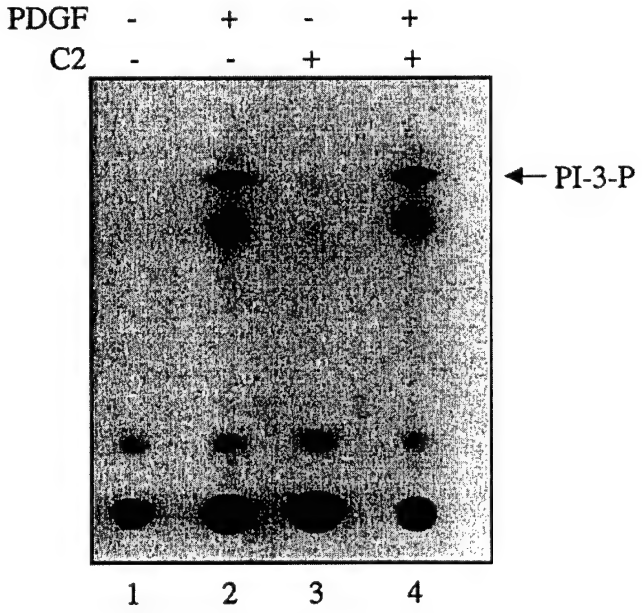

FIG. 3. Effect of C2 ceramide on PDGF-induced PI 3 kinase activity. Mesangial cells were treated with C2 followed by PDGF as described in Fig. 2. PDGFR immunoprecipitates from equal amounts of cell lysates were assayed for PI 3 kinase activity using PI as substrate as described under Materials and Methods. Arrow indicates the PI-3-phosphate product.

inhibitory effect of ceramide on PDGF-induced DNA synthesis in mesangial cells is not due to inhibition of PDGFR tyrosine kinase or PI 3 kinase. Rather, the inhibition of DNA synthesis by C2 may likely be due to post-PDGFR and post-PI 3 kinase events.

C2 ceramide inhibits PDGF-induced Akt serine threonine kinase activity. One of the downstream targets of PI 3 kinase is Akt (9). To examine the effect of C2 on Akt activation, we first investigated the effect of PDGF on its kinase activity in mesangial cells. Lysates of PDGF-stimulated mesangial cells were immunoprecipitated with an Akt antibody. Akt activity was determined in an immunecomplex kinase assay using histone H2B as substrate. PDGF increased Akt activity in a time-dependent manner (Fig. 4A). Maximum activation was observed 5 min after PDGF stimulation (Fig. 4A, lane 2). We have recently shown that PI 3 kinase regulates PDGF-induced DNA synthesis in mesangial cells (8). To determine the requirement of PI 3 kinase in Akt activation, we incubated mesangial cells with Ly 294002, a pharmacological inhibitor of PI 3 kinase, prior to incubation with PDGF. Akt activity was determined in an immune complex kinase assay. Ly 294002 significantly inhibited PDGF-induced Akt activity (Fig. 4B, compare lane 4 with lane 2) indicating that PI 3 kinase regulates PDGF-induced Akt serine threonine kinase activity in mesangial cells. Next we addressed the question whether C2 ceramide intercepts Akt to inhibit PDGF-induced DNA synthesis (Fig. 1). C2-treated mesangial cells were incubated with PDGF and the lysates were immunoprecipitated with Akt antibody followed by immune complex kinase assay. C2 completely inhibited PDGF-induced Akt activity (Fig. 4C, compare lane 4 with lane 2), just as it inhibited DNA synthesis (Fig. 1). These data indicate, for the first time, that Akt regulates PDGF-induced DNA synthesis in mesangial cells.

C2 ceramide blocks PDGF-induced c-fos gene transcription. As part of its mitogenic signaling, PDGF induces *de novo* transcription of early response genes, e.g., *c-fos* (44, 45). Previously it has been shown that PI

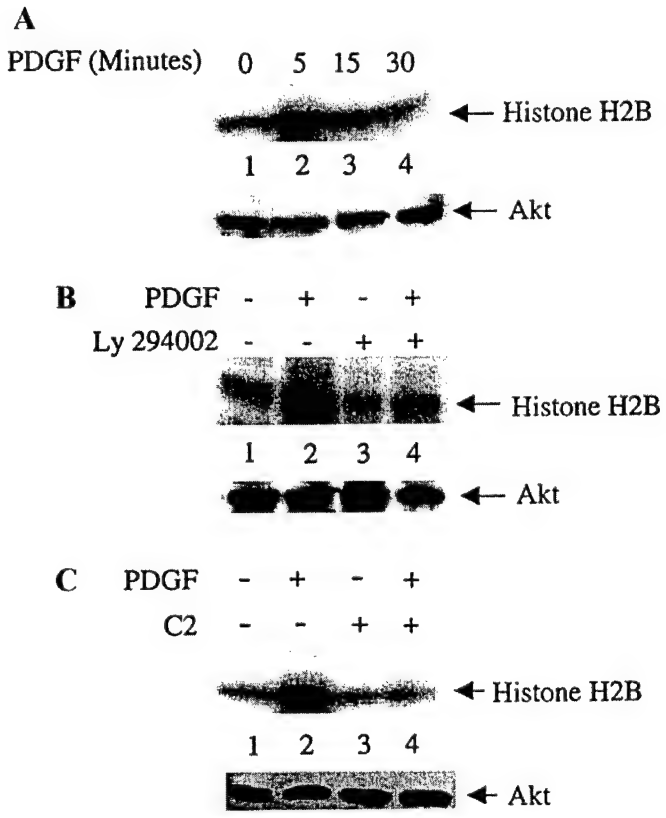

FIG. 4. C2 ceramide inhibits PDGF-induced Akt serine threonine kinase activity. (A) Kinetics of PDGF-induced Akt activation. Quiescent mesangial cells were incubated with 10 ng/ml of PDGF for different periods of time. 100 μg of cell lysate was immunoprecipitated with anti-Akt antibody. Akt immunoprecipitates were then used in an immunecomplex kinase assay with histone H2B as substrate, in the presence of [γ-^{32}P]ATP as described under Materials and Methods. The bottom panel shows immunoblot analysis of the same samples with Akt antibody. (B) Effect of PI 3 kinase inhibitor Ly 294002 on PDGF-induced Akt activity. Quiescent mesangial cells were incubated with 25 μM Ly 294002 for 1 h before stimulation with PDGF. Akt immunecomplex kinase assay was performed as described above. Bottom panel shows immunoblot analysis of the same samples with Akt antibody. (C) Effect of C2 ceramide on PDGF-induced Akt activity. Quiescent mesangial cells were incubated with 15 μM C2 before stimulation with PDGF. Akt immunecomplex kinase assay was performed with histone H2B as substrate as described under Materials and Methods. Bottom panel shows immunoblot analysis of the same samples with Akt antibody.

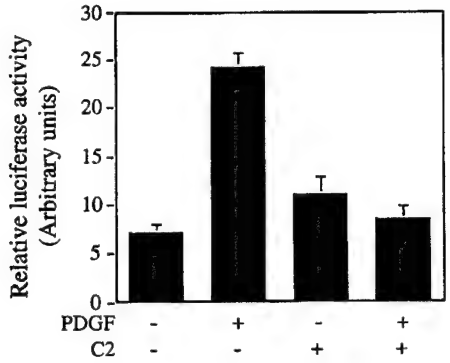

FIG. 5. Effect of C2 ceramide on PDGF-induced *c-fos* gene transcription. A reporter construct, in which the firefly luciferase cDNA is driven by a 550-bp fragment of *c-fos* promoter, was transiently transfected with CMV-Renilla plasmid into mesangial cells. Serum-deprived transfected cells were treated with C2 for 1 h followed by incubation with PDGF as described (35, 36). The luciferase activities were determined as described under Materials and Methods.

3 kinase regulates *c-fos* gene transcription (46). The mechanism of *c-fos* expression by PI 3 kinase, however, is not known. Since Akt is a downstream target of PI 3 kinase (9, 11) (Fig. 4B), and since C2 ceramide inhibits Akt activity (Fig. 4C) without any effect on PI 3 kinase activity (Fig. 3), we used this sphingolipid to address the regulation of *c-fos* gene transcription by Akt. A *c-fos* promoter-driven luciferase reporter construct was transfected into mesangial cells. As expected, PDGF stimulated reporter gene expression (Fig. 5). Incubation of these same reporter-transfected mesangial cells with C2 inhibited PDGF-induced reporter gene expression (Fig. 5). These data indicate that C2-mediated inhibition of PDGF-induced Akt kinase may be involved in regulating *c-fos* gene transcription induced by PDGF.

Effect of ceramide on mesangial cell death. One of the biological activities of ceramide is to induce apoptosis (20–22). This property is thought to be mediated in part by inhibition of the Akt serine threonine kinase (34, 43, 14–16). Since C2 ceramide inhibited PDGF-induced Akt activity (Fig. 4C), it may have induced apoptosis in mesangial cells. To examine the effect of C2 on apoptosis, mesangial cells were incubated with C2 ceramide for one hour followed by incubation with PDGF for 24 h. The cells were stained with Hoechst 33258, which only stains DNA in chromatin and allows distinction between apoptotic cells containing condensed chromatin and viable cells with normal chromatin structure (47). No condensed chromatin was observed with C2 alone or with C2 and PDGF-treated mesangial cells (Fig. 6). In contrast, incubation of mesangial cells with hydrogen peroxide, which was used as a positive control for inducing apoptosis, resulted in formation of condensed chromatin indicative of apoptosis (Fig. 6, indicated by dark white spots) (41, 47, 48).

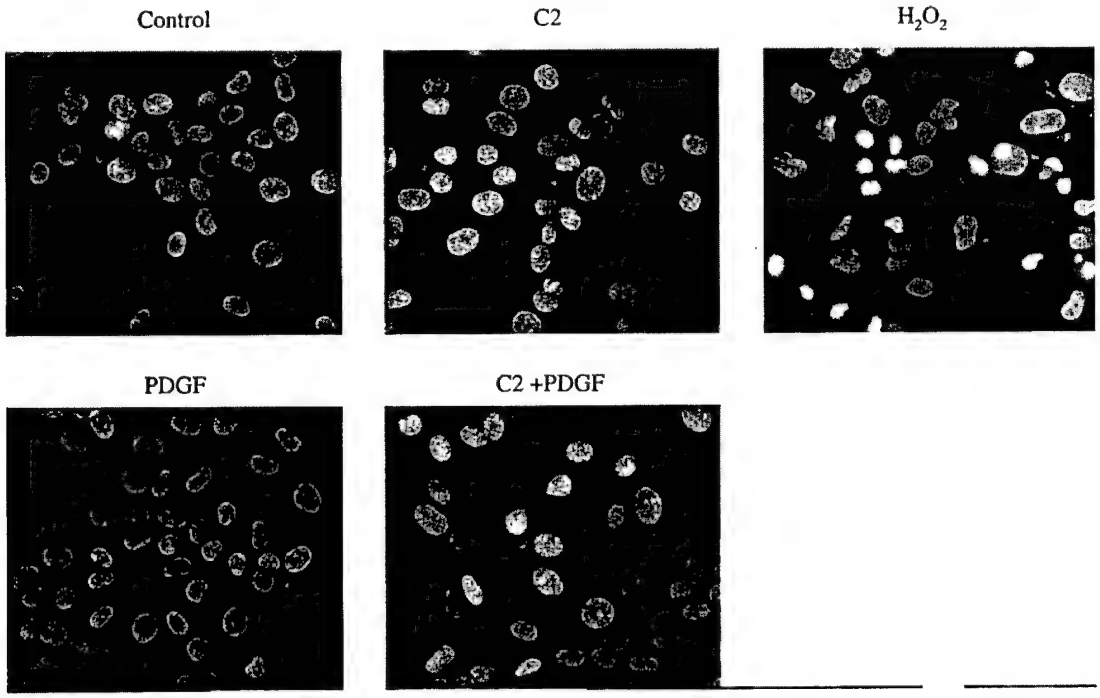

FIG. 6. Effect of C2 ceramide on mesangial cell apoptosis. Quiescent mesangial cells were incubated with 15 μM C2 for 1 h before stimulation with PDGF for 24 h. The cells were fixed with 4% paraformaldehyde and stained with Hoechst 33258, as described under Material and Methods. As a positive control for apoptosis, quiescent mesangial cells were incubated with 200 μM H_2O_2 for 24 h before staining with Hoechst 33258. Dark white spots in the H_2O_2-treated cells indicate condensed chromatin in the apoptotic cells.

To confirm this effect of C2, we stained C2-treated mesangial cells with propidium iodide. The stained cells were analyzed by confocal microscopy for chromatin condensation as a measure of apoptosis. No apoptosis was observed in C2-treated cells (data not shown). These data indicate that C2 ceramide does not induce apoptosis in mesangial cells. Therefore, the inhibition of PDGF-induced DNA synthesis observed in the presence of C2 (Fig. 1) was not due to apoptosis of mesangial cells.

DISCUSSION

This study provides the first evidence that C2 ceramide inhibits PDGF-induced DNA synthesis in mesangial cells by attenuating Akt serine threonine kinase activity, without causing any effect on PDGFR tyrosine kinase activity or PDGFR-associated PI 3 kinase activity. Furthermore, C2-dependent inhibition of PDGF-induced DNA synthesis was associated with inhibition of *c-fos* gene transcription. We have also demonstrated that C2-mediated inhibition of Akt activity does not induce apoptosis in these cells.

Proliferation of mesangial cells is a major pathological feature in many glomerular diseases (49). Increased expression of mesangial PDGFR in the glomeruli correlates with the proliferation of mesangial cells in animal models of mesangioproliferative glomerulonephritis (50). This increased activity of the PDGFR in disease states is reminiscent of the requirement of PDGFR function in mesangial cell development during embryogenesis (51, 52). PDGF stimulates three major signal transduction pathways, which include phospholipase Cγ1, mitogen activated protein kinase (MAPK) and PI 3 kinase (2, 5, 6). We previously reported that PDGF-induced MAPK activity is partially regulated by PI 3 kinase in mesangial cells (8). PI 3 kinase is the predominant pathway that regulates PDGF-induced DNA synthesis in these cells (8). PI 3 kinase regulates multiple signal transduction pathways that include activation of small GTP binding protein Rac, protein kinase C, ribosomal S6 kinase (RSK), p70S6 kinase (p70S6K), serum glucocorticoid kinase and Akt (11). Here we show that PDGF-stimulated PI 3 kinase utilizes its downstream target Akt serine threonine kinase for induction of DNA synthesis in mesangial cells.

At least 14 direct substrates of Akt have been identified in mammalian cells and include proteins that regulate apoptosis, protein synthesis and glycogen metabolism (4). The most extensively studied function of Akt, however, is its role in inhibition of apoptosis (13). For example the FKHR family of transcription factor induces expression of proapoptotic protein such as Fas ligand (16). Phosphorylation by Akt induces translocation of this transcription factor from the nucleus to the cytoplasm and results in inhibition of Fas ligand synthesis (16). It remains to be established whether this family of transcription factors is the target of Akt in mesangial cells. Similarly phosphorylation of caspase 9 by Akt inhibits its protease activity and prevents induction of apoptosis (15). Recently Fujita *et al.* reported that the phosphorylation of caspase 9 is species-dependent (53). For instance, caspase 9 from mouse or rat is not a substrate of Akt, due to lack of a consensus phosphorylation site (53). Therefore, Akt-dependent phosphorylation of caspase 9 may not be a major mechanism of cell survival in rat mesangial cells employed in this study.

A role for the PI 3 kinase/Akt pathway has been demonstrated in survival of cytokine-dependent hematopoietic cells as well as other cell types (13). In the case of interleukin-3-induced survival signaling, Akt stimulates phosphorylation of proapoptotic protein BAD resulting in its inactivation (54). But granulocyte/macrophage colony-stimulating factor maintains cell survival in the complete absence of Akt activity (55). On the other hand, IL-4 stimulates Akt kinase and cell survival but does not induce BAD phosphorylation (55). Furthermore, recent results in many different cell types indicate that BAD phosphorylation by Akt is not sufficient to inhibit apoptosis (55–57). In the present study, we provide evidence that in glomerular mesangial cells, inhibition of Akt activity by C2 ceramide does not induce apoptosis (Fig. 6), rather it inhibits PDGF-induced DNA synthesis (Fig. 1). This observation indicates that Akt may not initiate survival signals in mesangial cells. On the contrary, this PI 3 kinase target regulates the PDGF-induced mitogenic signal transduction pathway. These findings may have important therapeutic implications. Since PDGF-induced mesangial cell proliferation is a prominent feature of inflammatory glomerular diseases, identification and characterization of a safe Akt inhibitor may prove beneficial for these disorders.

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Bone Morphogenetic Protein-2 Inhibits MAPK-Dependent Elk-1 Transactivation and DNA Synthesis Induced by EGF in Mesangial Cells

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Bone morphogenetic protein-2 (BMP-2) is a member of the TGFβ superfamily of growth and differentiation factors. We investigated the effect of BMP-2 on epidermal growth factor (EGF)-induced mitogenic signaling in kidney glomerular mesangial cells. BMP-2 dose-dependently inhibits EGF-induced DNA synthesis. Maximum effect was obtained at a concentration of 100 ng/ml. BMP-2 had no inhibitory effect on the EGF receptor (EGFR)-associated tyrosine kinase activity indicating that inhibition of DNA synthesis is due to regulation of post-receptor signaling event(s). EGF stimulates MAPK activity in mesangial cells in a time-dependent manner. Inhibition of MAPK by the MEK inhibitor PD098059 blocks EGF-induced DNA synthesis indicating the requirement of this enzyme activity in EGF-mediated mitogenic signaling. Furthermore, we show that exposure of mesangial cells to BMP-2 blocks EGF-induced MAPK activity which leads to phosphorylattion of Elk-1 transcription factor. Using a GAL-4 DNA binding-domain-Elk-1 transactivation domain fusion protein-based reporter assay, we demonstrate that BMP-2 inhibits EGF-induced Elk-1-mediated transcription. These data provide the first evidence that BMP-2 signaling in mesangial cells initiates a negative regulatory cross-talk with MAPK-based transcription to inhibit EGF-induced DNA synthesis.

Bone morphogenetic proteins (BMPs) are a group of osteogenic factors and has recently been shown to play important role in the development of gut, heart, skin, teeth, lung and kidney (1). Several members of these proteins have been identified (1,2). Structurally, BMPs are highly homologous to TGFβ family of proteins (3,4). High affinity binding sites for BMP-2, a specific member of this family of proteins, has been identified in different cells and tissues including the kidney (5). BMPs exert their biologic effects via type II and type I serine threonine kinase receptors (3,4,6). Binding of BMP to the receptor stimulates oligomerization of the receptor complex resulting in phosphorylation of the type I receptor in the GS domain. Phosphorylated receptors then recruit the downstream signaling proteins Smad1, Smad 5 and Smad 8 among which Smad 1 is most extensively characterized (6,7). This receptor-associated Smad 1 is phosphorylated by the type I BMP receptor at the last two serine residues in the C-terminal SSVS sequence resulting in its translocation to the nucleus (8,9). It has recently been shown that the C-terminal segment of Smad1 codes for a transcriptional activation domain implying a role in gene expression (10).

EGF is a mitogen for many epithelial, neural and mesenchymal cells including glomerular mesangial cells, vascular pericytes that maintain the structural and functional integrity of the glomerular microvascular bed (11). Since biologically active EGF is a dimer, binding of EGF to intact cells results in dimerization of the receptor (12,13). Dimerization increases its intrinsic tyrosine kinase activity that autophosphorylates the cytoplasmic tail of the receptor at multiple tyrosine residues. These phosphotyrosines serve as the docking sites for many SH2-domain containing signaling proteins such as phosphatidylinositol 3 kinase (PI 3 kinase), phospholipase Cγ1 (PLCγ1), GTPase activating protein (GAP), Grb-2, Nck, Cbl and signal transducer and activator of transcription (STAT) (14-20). Mutational analysis of EGFR autophosphorylation sites

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demonstrated that for mitogenic activity, tyrosine phosphorylation of Shc, a PTB domain-containing adaptor protein, is sufficient (21). Shc recruits Grb-2/SOS signaling complex that activates the Ras/MAPK signal transduction pathways necessary for mitogenesis. It has recently been reported that activation of EGFR leads to phosphorylation of Smad 1, the downstream target of BMP, by a MAPK-dependent mechanism. This phosphorylation of Smad1 inhibits its localization in the nucleus thus blocking its transcriptional function (22).

In this report, we show that BMP-2 dose-dependently inhibits EGF-induced MAPK activity as well as EGF-induced DNA synthesis in mesangial cells. Inhibition of EGF-induced MAPK by BMP-2 is associated with inhibition of Elk-1-dependent gene transcription. Thus BMP-2 can act as a negative modulator of the MAPK/Elk-1 transcription pathway.

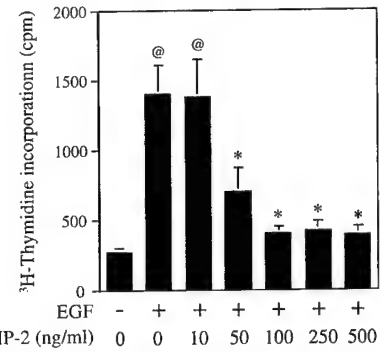

FIG. 1. Effect of BMP-2 on EGF-induced DNA synthesis in human mesangial cells. Confluent cells in 24-well dishes were serum-deprived and treated with different concentration of BMP-2 for 30 minutes. EGF at a concentration of 50 ng/ml was added. ^{3}H-thymidine incorporation was determined as a measure of DNA synthesis as described (23,25). Results are means ± SE of 3 independent experiments each done in triplicate. @$P < 0.05$ vs EGF-untreated cells. *$P < 0.05$ vs EGF-treated but BMP-2 untreated cells.

MATERIALS AND METHODS

Materials. Tissue culture reagents were purchased from Gibco/BRL (Rockville, MD). EGF was from R & D (Minneapolis, MN). EGFR antibody was obtained from Oncogene Science (Cambridge, MA). The MAPK antibody which recognizes Erk1 and to a lesser extent Erk2 was purchased from Santacruz (CA). Protein A sapphires CL4B was obtained from Pharmacy LAB Biotechnology Inc (Piscataway, NJ). γ^{32}P-ATP was from NEN (Boston, MA). Protein measurement and polyacrylamide gel reagents were purchased from Bio-Rad (Hercules, CA). GAL-4-Elk-1 fusion plasmid and GAL-4-luciferase reporter plasmid were obtained from Stratagene (La Jolla, CA). Lipofectamine plus reagent was purchased from Life Technology (Grand Island, NY). Dual luciferase kit was obtained from Promega (Madison, WI). All other reagents were obtained from Sigma (St. Louis, MO).

Cell culture. Rat glomerular mesangial cells (kindly provided by Dr. Jeff Kreisberg, Department of Pathology, University of Texas Health Science Center at San Antonio) and human mesangial cells were isolated and characterized as described (23,24) and used between passages 15th and 25th and 6th and 10th respectively. Cells were serum deprived for 48 hours and treated with BMP-2 for 30 minutes before addition of EGF. For ease of transfection of rat mesangial cells, transfections and biochemical studies were carried out in these cells.

Measurement of DNA synthesis. DNA synthesis in human mesangial cells was measured as ^{3}H-thymidine incorporation into trichloroacetic acid insoluble material as described previously (23,25).

Immunoprecipitation, tyrosine kinase, and MAPK assay. The cells were lysed in solubilization buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF and 0.1% aprotinin). Cleared cell lysate was immunoprecipitated with EGFR specific antibody and EGFR immunoprecipitates were washed three times with solubilization buffer and twice with 50 mM Tris-HCl, pH 7.4, 0.5 mM Na_3VO_4 . The immunebeads were then used in an *in vitro* tyrosine kinase assay as described previously (26,27). For MAPK activity, MAPK immunoprecipitates were assayed in the presence of myelin basic protein (MBP) and γ^{32}P-ATP as described previously (23). The phosphorylated MBP was separated by 15% SDS polyacrylamide gel electrophoresis.

Immunoblotting. Immunoblotting of MAPK was performed as described previously (25,28).

Transient transfection and luciferase activity. Transient transfections of mesangial cells were performed with lipofectamine plus reagent as suggested by vendor. Briefly 1 μg of firefly luciferase reporter plasmid and GAL-4-DNA binding domain Elk-1 transactivation domain fusion plasmids were cotransfected with 12 ng of CMV-Renilla luciferase reporter plasmid into mesangial cells. The cells were then grown to confluency and serum deprived for 48 hours before stimulation with EGF. The luciferase activity in the cell lysate was determined and corrected using the dual luciferase assay kit as suggested by the vendor.

RESULTS

BMP-2 inhibits EGF-induced DNA synthesis. We and others have previously shown that EGF stimulates mesangial cell proliferation (11,29). We also reported that TGFβ inhibits EGF-induced DNA synthesis (30). BMP-2 belongs to the TGFβ superfamily. We studied the effect of BMP-2 on EGF-induced DNA synthesis in mesangial cells. The cells were incubated with different concentrations of BMP-2 prior to treatment with EGF. ^{3}H-thymidine incorporation was determined as a measure of DNA synthesis. As shown in the Fig. 1, EGF stimulates DNA synthesis in these cells and 50 ng/ml BMP-2 results in significant inhibition of DNA synthesis. Approximately 70% inhibition of DNA synthesis was obtained with 100 ng/ml of BMP-2. No further inhibition was obtained even at a dose of 500 ng/ml BMP-2.

Effect of BMP-2 on EGFR tyrosine kinase activity. EGF stimulates its receptor tyrosine kinase activity to exert its biological effects in target cells. It was reported earlier that expression of kinase-deficient mutant of EGFR blocks EGF-induced DNA synthesis (31). Thus the inhibitory effect of BMP-2 on EGF-indcued DNA synthesis may be a result of inhibition of EGFR tyrosine kinase activity. To test the effect of BMP-2 on EGFR tyrosine kinase activity, mesangial cells were

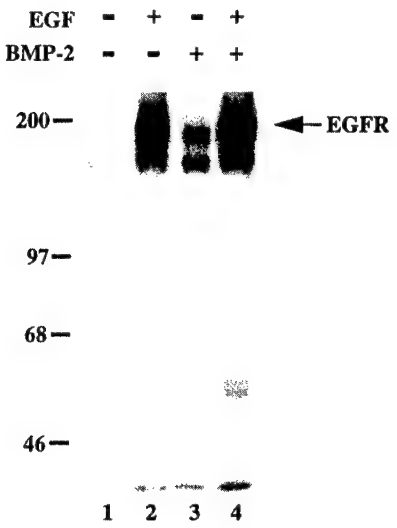

FIG. 2. Effect of BMP-2 on EGFR tyrosine kinase activity. Serum-deprived mesangial cells were treated with 100 ng/ml BMP-2 for 30 minutes before incubation with EGF for 5 minutes. The cleared cell lysate was immunoprecipitated with anti-EGFR monoclonal antibody and the immunobeads were assayed for EGFR-associated tyrosine kinase activity in the presence of γ^{32}P-ATP as described in the methods. The autophosphorylated EGFR was separated by 7.5% SDS polyacrylamide gel electrophoresis and visualized by autoradiography. The arrow indicates the fully mature EGFR, while the faster migrating band is the unprocessed protein. Molecular weight markers are shown in the left margin in kDs.

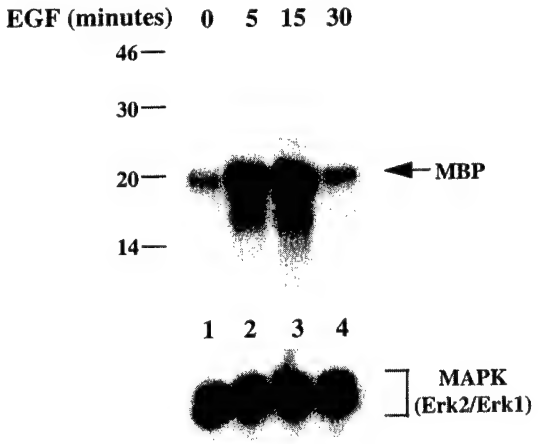

FIG. 3. Effect of EGF on MAPK activity in mesangial cells. Serum-deprived cells were treated with EGF for different periods of time. 100 μg of lysate was immunoprecipitated with MAPK specific antibody and the immunoprecipitates were used in an *in vitro* MAPK assay in the presence of MBP as substrate and γ^{32}P-ATP. Phosphorylated MBP was separated on a 15% SDS gel and visualized by autoradiography. The molecular weight markers in kDs are shown in left margin. Bottom panel shows the immunoblot analysis of cell lysates with MAPK antibody.

incubated with BMP-2 prior to EGF treatment. The cell lysate was immunoprecipitated with EGFR antibody and the immunoprecipitates were used to assay the receptor-associated tyrosine kinase activity. As expected, EGF stimulates the tyrosine kinase activity of EGFR (Fig. 2, compare lane 2 with 1). However, incubation of cells with BMP-2 does not inhibit EGF-induced tyrosine kinase activity (compare lane 4 with 2). These data indicate that BMP-2 does not regulate EGFR autophosphorylation but rather it may alter downstream signaling molecule(s) of the receptor signal transduction.

MAPK regulates EGF-induced DNA synthesis in mesangial cells. Activation of MAPK has been shown to be an important step in modulating mitogenic signals for many growth factors and cytokines (32). However, the requirement of MAPK in EGF-induced DNA synthesis in mesangial cells has not been explored. To study this, we first investigated the kinetics of MAPK activation in mesangial cells. The cells were incubated with EGF for different periods of time and the cell lysates were immunoprecipitated with MAPK specific antibody. The immunebeads were used in an immunecomplex kinase assay in the presence of MBP and γ^{32}P-ATP. The results show a time-dependent increase in MAPK activity in response to EGF (Fig. 3). To study the requirement of MAPK for EGF-mediated mitogenic signaling, we used the compound PD098059 which blocks the activity of MEK, the upstream activating kinase of MAPK, thus resulting in inhibition of MAPK. We first tested the effect of PD098059 on EGF-induced MAPK activity in mesangial cells. The cells were incubated with PD098059 followed by treatment with EGF. The cell lysate was then used to immunoprecipitate MAPK and its activity was measured in an immunecomplex kinase assay. The data show that MEK inhibitor blocks EGF-induced MAPK activity (Fig. 4).

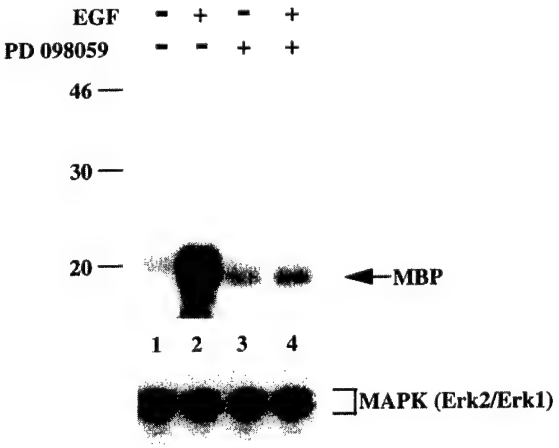

FIG. 4. Effect of MEK inhibitor PD098059 on EGF-induced MAPK activity. Serum-deprived mesangial cells were treated with 25 μM PD 098059 for one hour before the addition of EGF for 5 minutes. MAPK activity was measured in the MAPK immunoprecipitates as described in Fig. 3. Molecular weight markers in kDs are shown in left margin. Bottom panel shows the immunoblot analysis of cell lysates with MAPK antibody.

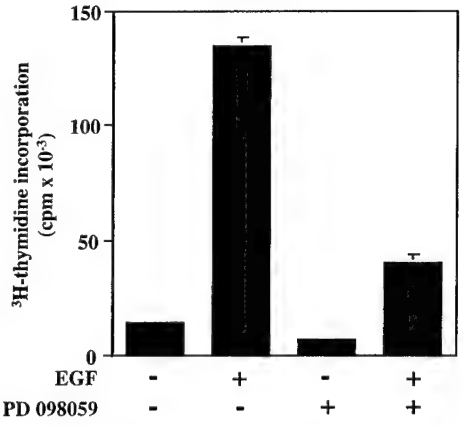

FIG. 5. Effect of MEK inhibitor on EGF-induced DNA synthesis in mesangial cells. Serum-deprived cells were treated with 25 μM PD098059 for one hour before the addition of EGF. DNA synthesis was determined as described in Fig. 1.

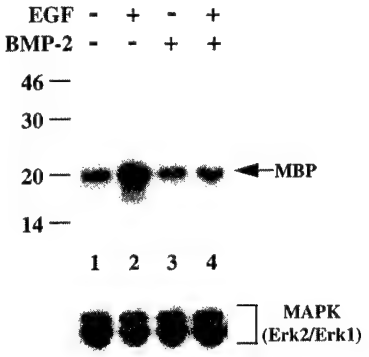

FIG. 6. Effect of BMP-2 on EGF-induced MAPK activity in mesangial cells. Serum-deprived cells were treated with 100 ng/ml BMP-2 for 30 minutes before addition of EGF. MAPK activity was measured in the immunoprecipitates as described in Fig. 3. Molecular weight markers in kDs are shown in left margin. Bottom panel shows the immunoblot analysis of cell lysates with MAPK antibody.

Next we studied the effect of PD098059 on EGF-induced DNA synthesis. As shown in Fig. 5, incubation of mesangial cells with PD098059 inhibits EGF-induced DNA synthesis by 70% similar to that observed by BMP-2 (Fig. 1).

BMP-2 inhibits EGF-induced MAPK activity. We have established that MAPK regulates EGF-mediated mitogenic signaling in mesangial cells (Fig. 5). We have also shown that BMP-2 inhibits EGF-induced DNA synthesis in these cells in a dose-dependent manner without affecting the tyrosine kinase activity of its receptor (Fig. 1 and Fig. 2). Therefore, to test whether the inhibitory effect of BMP-2 is mediated by interception of MAPK induced by EGF, we incubated mesangial cells with BMP-2 followed by treatment with EGF. MAPK activity was measured in MAPK immunoprecipitates from the lysates of these cells by immunecomplex kinase assay. The data show complete inhibition of EGF-induced MAPK activity by BMP-2 (Fig. 6). These results indicate that BMP-2-induced inhibition of DNA synthesis stimulated by EGF is due to attenuation of MAPK activity.

BMP-2 inhibits EGF-induced Elk-1-mediated transcription. It is established that activation of MAPK leads to its translocation to the nucleus where it phosphorylates transcription factors necessary for cell function (33). One of the target transcription factor of MAPK is the ETS family protein Elk-1 (34). It has been shown that addition of mitogen such as serum to cultured cells stimulates phosphorylation of Elk-1 in serine residues in its C-terminus. This phosphorylation is necessary for its transactivation function and gene expression (35). First, we studied the role of MAPK in EGF-induced Elk-1-mediated gene transcription. We cotransfected into mesangial cells an expression vector encoding the Elk-1 C-terminal transactivation domain fused to yeast GAL-4 DNA binding domain and a firefly luciferase reporter plasmid under the control of GAL-4 DNA element. Addition of EGF to these cells increases GAL-4-dependent expression of the reporter gene (Fig. 7). Prior exposure of these transfected mesangial cells to the MEK inhibitor PD098059 blocks EGF-induced Elk-1-mediated transcription (Fig. 7). To test the effect of MAPK directly on Elk-1 transactivation, we cotransfected an expression vector coding for a constitutively active form of MEK that activates MAPK along with the GAL 4-Elk-1 fusion plasmid and the reporter construct. Coexpression of constitutively active MEK stimulated Elk-1-mediated transcription of reporter gene (data not shown). To further confirm the effect of MAPK on Elk-1, we cotransfected a dominant negative form of Erk2 MAPK along with the

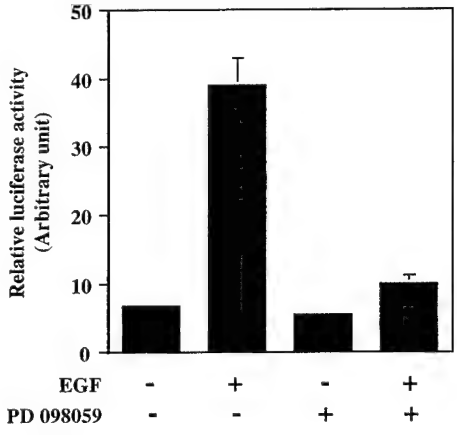

FIG. 7. Inhibition of MAPK blocks EGF-induced Elk-1-mediated transcription of reporter gene. Mesangial cells were cotransfected with 1 mg of GAL-4-luciferase reporter plasmid and GAL-4-Elk-1 fusion plasmid along with 12 ng of CMV-Renilla plasmid as described in Methods. Serum-deprived transfected cells were treated with PD098059 for one hour before addition of EGF. The luciferase activity was measured as described in the Methods.

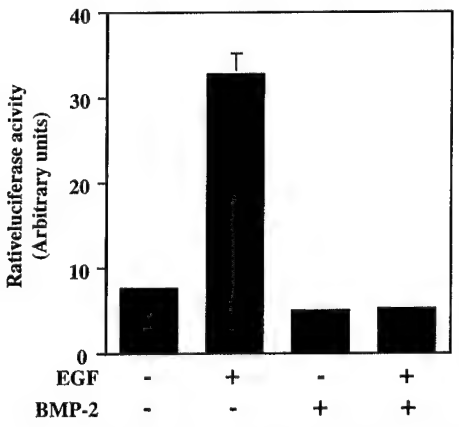

FIG. 8. Effect of BMP-2 on EGF-induced Elk-mediated transcription. Mesangial cells were transfected with the same plasmids as described in Fig. 7. The cells were then serum starved for 48 hours and treated with BMP-2 for 30 minutes before addition of EGF. The cell lysates were used for luciferase activity as described in the Methods.

Elk-1 and reporter constructs. The results showed inhibition of Elk-1 transactivation by expression of dominant negative MAPK (data not shown). These data indicate that inhibition of MAPK blocks Elk-1-mediated transcription. We have shown above that BMP-2 inhibits EGF-induced MAPK activity. Therefore we tested effect of BMP-2 on EGF-induced transactivation of Elk-1. Mesangial cells were cotransfected with GAL-4 Elk-1 fusion plasmid and GAL-4 reporter plasmid. These transiently transfected cells were treated with BMP-2 followed by incubation with EGF. Luciferase activity was determined as a measure of Elk-1 transactivation. As shown in Fig. 8, BMP-2 inhibits EGF-induced transactivation of Elk-1. These data indicate that BMP-2 blocks Elk-1-dependent transcriptional events via its inhibition of MAPK induced by EGF.

DISCUSSION

Accumulating evidence indicates that proliferation of mesangial cells play important role in glomerular disorders (36). Previous studies have shown that EGF stimulates DNA synthesis in mesangial cells (29). This study demonstrates that BMP-2 inhibits EGF-induced DNA synthesis by blocking EGF-induced MAPK activity. BMP-2 does not inhibit EGFR tyrosine kinase activity. However, BMP-2 attenuates EGF-induced activation of Elk-1 transcription factor. These data provide the first evidence that BMP-2 intercepts EGF-induced MAPK-dependent transcriptional events to attenuate proliferative signals.

TGFβ and BMPs are members of same superfamily of proteins. Both TGFβ and BMPs function through distinct type I and type II serine threonine kinase receptors. In the case of TGFβ, the ligand binds to type II receptor and then recruits and phosphorylates type I receptor to undergo oligomerization and increase its intrinsic serine/threonine kinase activity (3,4). BMPs bind to these receptors independently with low affinity. However, in the presence of both, BMPs can bind with very high affinity that initiates the induction of necessary genes required for the biologic activity. We previously reported that TGFβ inhibits EGF-induced DNA synthesis in mesangial cells (30). We now show that similar to TGFβ, BMP-2 also inhibits EGF-induced DNA synthesis in these cells (Fig. 1).

EGFR is known to be phosphorylated at serine residues by downstream kinases (37). Phosphorylation of EGFR on serine residues causes inactivation of its tyrosine kinase activity with concomitant inhibition of its mitogenic capacity (37,38). Thus in the present study, the EGFR may be negatively regulated by serine phosphorylation via BMP receptor signaling when mesangial cells are exposed to BMP-2. However, our data directly argue against this notion since BMP-2 does not inhibit EGF-induced tyrosine kinase activity of the EGFR in mesangial cells (Fig. 2).

It has been shown that tyrosine kinase activity of the EGFR is necessary for its mitogenic action (31). Tyrosine phosphorylated EGFR physically interacts with Grb-2-SOS complex at tyrosine 1068 at a very high affinity resulting in the recruitment of SOS in the plasma membrane to induce exchange of GTP for GDP in Ras (17,39). Thus EGFR can directly bind Grb-2-SOS complex and results in activation of the Ras/MAPK pathway necessary for DNA synthesis (17). In addition, activation of mutant EGFR in which all five autophosphorylation sites are mutated and lacks binding site for Grb-2 directly, leads to phosphorylation of Shc which associates with Grb-2-SOS complex, thus indirectly activating Ras/MAPK pathway. Such autophosphorylation mutant of EGFR that lacks the binding sites for other signaling proteins retains its ability to stimulate DNA synthesis (21). Thus, activation of MAPK in response to EGFR by differrent pathways correlates with its ability to stimulate DNA synthesis in these cells. It should be emphasized that Ras-mediated activation of MAPK has been shown to be cell-type specific (40,41). In the present study, we demonstrate that in mesangial cells, EGF-induced DNA synthesis is predominantly mediated by MAPK. Inhibition of MAPK resulted in approximately 70% inhibition of DNA synthesis (Fig. 5) which is similar to the inhibition caused by BMP-2 (Fig. 1). The inability of BMP-2 to fully inhibit DNA synthesis may be due to contribution from other mitogenic signal transduction pathways such as PLCγ1 and PI 3 kinase which are also downstream targets and have been shown to be involved in growth factor-induced DNA synthesis (42). Thus BMP-2 may not completely inhibit all the early parallel mitogenic signal transduction pathways induced by EGF.

BMP-2 stimulates growth and differentiation of osteogenic cells (2). We have recently shown that in preosteoblastic cells, BMP-2 activates MAPK (43). Inhibition of MAPK activity by the MEK inhibitor PD098059 blocks growth and differentiation of these cells (43). Therefor, MAPK may positively regulate the biological effects of BMP-2 in these cells. There are several examples of BMP receptor and tyrosine kinase receptor cross-talk during growth and differentiation. Thus fibroblast growth factor (FGF) receptor tyrosine kinase inhibits the antiproliferative effect of BMP-2 during outgrowth of limb bud (44). Another example is the opposing effect of EGFR activation on BMP-2-induced expression of osteogenic markers (45). BMP-2 also blocks the expression of genes necessary for tooth development by FGF (46). Thus negative cross-talk exists between receptor tyrosine kinase and the BMP receptor serine/threonine kinase and/or its downstream components. We demonstrate here that BMP-2 inhibits EGF-induced MAPK activity in mesangial cells. MAPK is primarily regulated by Ras, Raf 1 and MEK (32,39). Also protein kinase C stimulates MAPK in mesangial cells (data not shown). EGF is known to activate all these enzymes. Which of these proteins are targeted by BMP-2 to induce the inhibitory effect is not yet known.

MAPK regulates the activity of several transcription factors (33-35,47). In *Caenorhabditis elegans,* activation of let 23, the mammalian homolog of EGFR, stimulates MPK-1 which phosphorylates the winged-helix transcription factor LIN-31. This phosphorylation dissociates LIN-31 from its inhibitory counterpart LIN-1, an ETS domain transcription factor that suppresses vulval development in *C. elegans* in the absence of phosphorylation (48). Also LIN-1 is a substrate for MAPK *in vitro* and *in vivo* (48). However, the functional consequences of this phosphorylation is not known yet. In Drosophila melanogaster, the MAPK homolog Rolled/ERKA phosphorylates the ETS domain transcription factors Pointed P2 and YAN in R7 photoreceptor cells during eye development. Phosphorylation of YAN negatively regulates its repressor function while phosphorylation of Pointed P2 activates the transcription of target genes necessary for R7 cell development (49). In mammalian cells, MAPK phosphorylates the ETS domain transcription factor Elk-1 in al least three serine residues in the C-terminal C-box (32,35). MAPK phosphorylates the consensus PXS/TP sites although a minimal consensus sequence S/TP which is the most critical serine 383 phosphorylation site in Elk-1, is sufficient for phosphorylation (50). Phosphorylation of this minimal consensus site has been shown to be dependent upon the presence of D-domain, the MAPK targeting domain, present N-terminal to the C-domain in Elk-1 (51). Serum stimulation of cells activate MAPK that translocates to the nucleus and binds to Elk-1 D-domain to phosphorylate at least three serine residues in the C-terminal C-box (32,35). Mutation of specific residues in MAPK binding sites or mutation of MAPK phosphorylation sites in Elk-1 reduces EGF or serum-induced transcriptional activation of a reporter gene (34,51,52). We now provide evidence that inhibition of EGF-induced MAPK activity by PD098059 or BMP-2 blocks GAL-4-Elk-1-mediated transcription in mesangial cells (Fig. 7 and Fig. 8). These data indicate that in these cells, Elk-1 mediated transcription may be a necessary component of the mitogenic effect of EGF.

Mesangial cell proliferation is a prominent feature of many inflammatory glomerular diseases. Although BMP receptor is abundantly expressed in mesangial cells, the role of BMP-2 in the biology of these and other kidney cells remains to be determined. Our observation that BMP-2 inhibits DNA synthesis in mesangial cells suggests that it may prove to be an effective antiproliferative agent for mesangioproliferative disorders.

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Bone Morphogenetic Protein-2 Blocks MDA MB 231 Human Breast Cancer Cell Proliferation by Inhibiting Cyclin-Dependent Kinase-Mediated Retinoblastoma Protein Phosphorylation

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Bone morphogenetic protein-2 (BMP-2) has been shown to act as an antiproliferative agent for a number of different cell types. We show that BMP-2 dose-dependently inhibits growth of MDA MB 231 human breast cancer cells. Epidermal growth factor (EGF) stimulates DNA synthesis and entry of these cells into the S-phase. BMP-2 inhibits EGF-induced DNA synthesis by arresting them in G1 phase of the cell cycle. BMP-2 increases the level of cyclin kinase inhibitor p21. Furthermore, we show that exposure of MDA MB 231 cells to BMP-2 stimulates association of p21 with cyclin D1 and with cyclin E resulting in the inhibition of their associated kinase activities. Finally, BMP-2 treatment is found to cause hypophosphorylation of the retinoblastoma protein (pRb), a key regulator of cell cycle progression. Our data provide a mechanism for the antiproliferative effect of BMP-2 in the breast cancer cells. © 2000 Academic Press

Key Words: **BMP-2; pRb; p21; breast cancer cells.**

The growth of breast cancer cells is regulated by a variety of steroid hormones and growth factors. In the late stage of hormone-dependent tumor cell growth, the cells become hormone-independent. One such human breast tumor cell line, MDA MB 231 (MDA), derived from a metastasized human adenocarcinoma, is estrogen-independent (1). However, they maintain the responsiveness to growth factors, such as epidermal growth factor (EGF) (2). The proliferative signals generated in the cytosol integrate into the nucleus to activate the cyclins and cyclin-dependent kinases (CDKs) that regulate the cell cycle progression of breast tumor cells (3). Thus in the early G1 phase of cell cycle, the D type cyclin activates the CDK 4 while in the mid and late G1, cyclin E and CDK2 are activated. These kinases also remain activated during S-phase of cell cycle (4, 5). These kinases phosphorylate the retinoblastoma tumor suppressor retinoblastoma protein (pRb) to drive the cells through the cell cycle (6). However, the cyclin kinase inhibitor proteins, such as p21, interacts with the cyclins thus inhibiting the CDKs resulting in blockage of cell cycle (7–9). Many agents that inhibit cell proliferation regulate the expression and activity of this group of proteins (7).

Bone morphogenetic proteins are structurally similar to the TGF β super family (10). Bone morphogenetic protein-2 (BMP-2), a member of this large family of proteins, stimulates growth and differentiation of osteogenic and chondrogenic cells during bone remodeling and also plays an important role in embryogenesis (10–13). Similar to TGF β, BMPs exert their effect via specific type I and type II serine-threonine kinase receptors. Binding of BMP-2 to the type II receptor induces the oligomerization of the receptor complex resulting in phosphorylation of the type I receptor and recruitment of downstream signaling proteins Smad 1, Smad 5 and Smad 8 (14, 15). Among these, Smad 1 has been extensively studied as the target of BMPR signaling. Type I BMPR-phosphorylated Smad1 then heterodimerizes with Smad 4 and translocates to the nucleus to act as a transcription factor and induce genes that mediate the biological activity of BMP-2 (16).

BMP-2 has recently been reported to have a growth inhibitory effect on prostate cancer cells (17). BMP-2 also inhibits smooth muscle cell proliferation (18). We have recently shown that BMP-2 inhibits PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells by inhibiting mitogen activated protein

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kinase (MAPK) cascade (19, 20). A very high dose of BMP-2 inhibits soft agar growth of a variety of tumor samples including breast tumor (21). However the mechanism of inhibition of tumor cell proliferation by BMP-2 is not yet clear.

In this study we demonstrate that BMP-2 inhibits growth of MDA MB 231 human breast cancer cells in culture in the presence and in the absence of EGF. BMP-2 increases p21 cyclin kinase inhibitor in these cells and inhibits cyclin D1- and E-associated kinases. Furthermore BMP-2 inhibits pRb phosphorylation, which results in accumulation of cells in the G1 phase of cell cycle resulting in inhibition of DNA synthesis.

MATERIALS AND METHODS

Materials. Tissue culture reagents were purchased from Gibco/BRL (Rockville, MD). EGF was from R & D (Minneapolis, MN). Phospho pRb antibody was purchased from New England Biolabs. All other antibodies, GST-pRB and Protein A/G plus were obtained from Santa Cruz. Protein A–Sepharose CL 4B was purchased from Pharmacia. Histone H1 was purchased from Sigma. ECL reagent was purchased from Pierce laboratories. Recombinant BMP-2 was obtained from Genetics Institute.

Cell culture. MDA MB 231 cells were grown in IMEM with 5% fetal bovine serum. For experiments the cells were grown in complete medium for 48 h and serum deprived for 24 h before addition of 100 ng/ml EGF. EGF causes modest proliferative response in this isolate of MDA MB 231 cells. For cell cycle analysis, near confluent cells were used for 24 h serum-deprivation to arrest in G0/G1 phase before addition of EGF to release them. To detect the effect of EGF on MDA MB 231 cell proliferation, serum free medium was changed every 6 h.

Measurement of DNA synthesis. DNA synthesis was measured as ^{3}H-thymidine incorporation into trichloroacetic acid insoluble material as described previously (19, 20).

Flow cytometric analysis. Trypsinized MDA MB 231 cells were washed with PBS and fixed in 70% ethanol for 30 min at −20°C. The cells were then centrifuged at 1500 × g for 4 min, washed with PBS containing 1% BSA and resuspended in 150 μl PBS. For nuclear staining, the cells were treated with 50 μl of 1 mg/ml RNase A followed by 100 μl of 100 μg/ml propidium iodide and incubated at 4°C for 24 h. The cells were then analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA) using 200 mW of light at 488 nm produced by an argon-ion laser. The fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20,000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

Immunoprecipitation and immunoblotting. The cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF and 0.1% aprotinin) for 30 min at 4°C. The cleared cell lysate was immunoprecipitated with required antibody essentially as described previously (19, 20). Immunoblotting of the immunoprecipitates or the cell lysate was also performed as described previously (19, 20, 22).

Cyclin D1- and E-associated kinase activity. The assay was performed according to the method of Gong et al (23). Briefly, cell lysates were immunoprecipitated with cyclin D1 or cyclin E antibody. The immunebeads were resuspended in kinase assay buffer (20 mM Tris-HCl, pH 7.5 and 4 mM $MgCl_2$). For cyclin D1-associated kinase assay a fragment of pRb containing the *in vivo* phosphorylation sites was used as substrate. For cyclin E-associated kinase activity, histone H1 was used as substrate. The reaction was carried out with 25 μM ATP containing 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reaction product was separated by SDS–polyacrylamide gel electrophoresis and the phosphorylated proteins were visualized by autoradiography.

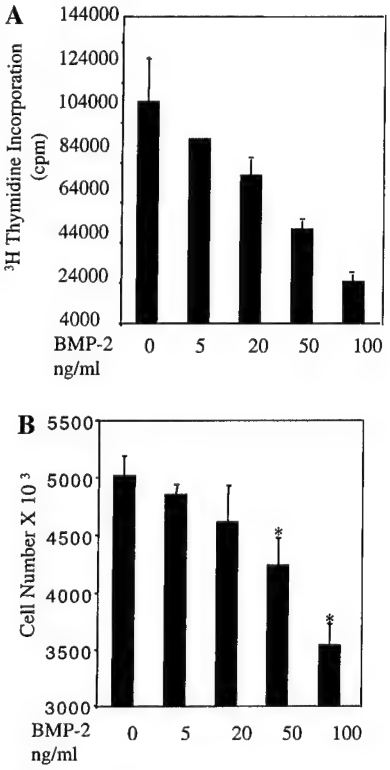

FIG. 1. (A) Effect of BMP-2 on DNA synthesis in MDA MB 231 cells. Serum-deprived MDA MB 231 cells were incubated with different concentrations of BMP-2 for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis, as described under Materials and Methods (19, 20). (B) Effect of BMP-2 on MDA MB 231 cell proliferation. Subconfluent cultures of MDA MB 231 cells in triplicate dishes were incubated with different concentrations of BMP-2 for 24 h. Number of cells were counted for each treatment condition. Mean ± SE of three independent experiments. *$P < 0.05$ vs untreated cells.

RESULTS

BMP-2 inhibits MDA MB 231 cell proliferation. MDA MB 231 cells grow very aggressively in culture. BMP-2 has recently been shown to have an antiproliferative effect on certain cell lines, including primary mesangial cells and prostate cancer cells (17, 19, 20). We examined the effect of BMP-2 on DNA synthesis in MDA MB 231 cells. MDA MB 231 cells were incubated with increasing concentrations of BMP-2 for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis in these cells. Figure 1A shows that BMP-2 inhibits DNA synthesis in a dose-dependent manner. Approximately 75% inhibition in DNA synthesis was observed at 100 ng/ml BMP-2. To

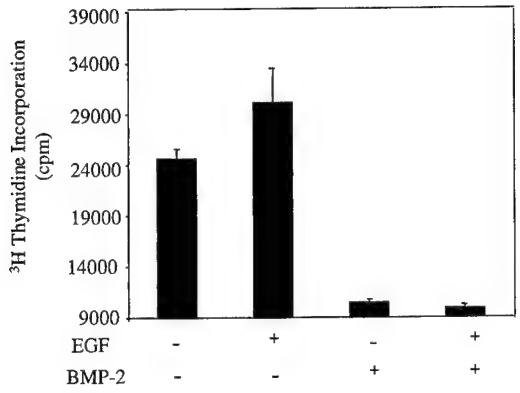

FIG. 2. Effect of BMP-2 on EGF-induced DNA synthesis. Serum-deprived MDA MB 231 cells were incubated with 100 ng/ml BMP-2 for 30 min followed by 100 ng/ml EGF for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis, as described under Materials and Methods (19, 20).

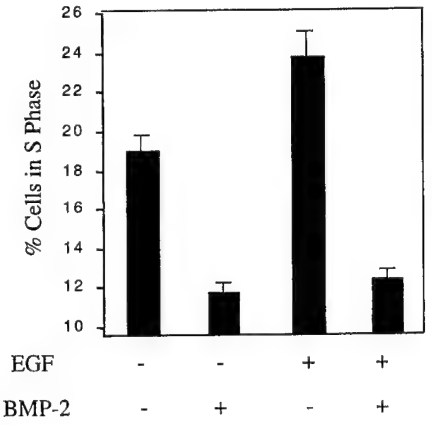

FIG. 3. S-phase analysis of EGF-induced MDA MB 231 cells. Serum-deprived MDA MB 231 cells were incubated with BMP-2 for 30 min and treated in the presence or absence of 100 ng/ml EGF for 24 h. Cells were then trypsinized and analyzed by flow cytometry as described under Materials and Methods. The percentage of cells in S-phase was plotted. Means of triplicate determinations are shown.

confirm this observed effect of BMP-2, we studied the effect of BMP-2 on MDA MB 231 cell proliferation in culture. Growing MDA MB 231 cells were treated with increasing concentrations of BMP-2. The cell number was determined. The data show that BMP-2 dose dependently inhibits MDA MB 231 cell growth in 24 h (Fig. 1B). As evident, 100 ng/ml BMP-2 inhibited proliferation of MDA MB 231 cells by 70% as compared to the untreated control. These data indicate that BMP-2 dose-dependently inhibits proliferation of MDA-MB-231 cells.

Inhibition of EGF-induced growth of MDA MB 231 cells by BMP-2. Activation of EGF receptor family is often associated with breast cancer cell growth (24). To test the effect of BMP-2 on EGF-induced DNA synthesis in MDA MB 231 cells, [^{3}H]thymidine incorporation was determined in the presence and absence of BMP-2. As shown in Fig. 2, EGF increases the DNA synthesis in MDA MB 231 cells by 20%. However, BMP-2 inhibits DNA synthesis by 58% in these cells in the presence of EGF. Since the increase in DNA synthesis by EGF was modest, we tested the effect of EGF on MDA MB 231 cell cycle progression using FACS analysis. Using this technique, EGF increased the number of cells in S phase by 31% (Fig. 3). These data indicate that the mitogenic effect of EGF on MDA MB 231 cells is due to increased progression of these cells from G1 to S phase. Incubation with BMP-2 shows a 58% decrease of cells in the S-phase (Fig. 3). This decrease in the S phase population is accompanied by an increase in cell number at the G1 phase of cell cycle (Table 1). These data indicate that BMP-2 inhibits EGF-induced MDA MB 231 cell proliferation by arresting them at the G1 phase of cell cycle.

BMP-2 induces CDK inhibitor p21 in MDA MB 231 cells. The key proteins that regulate cell cycle progression from G1 to S phase are cyclins D and E and the CDKs that are associated with these cyclins (4, 5). The presence of cyclin kinase inhibitor such as p21 causes cell cycle arrest in G1 by quenching cyclin D and E resulting in inhibition of their associated kinase activity (7–9). Since BMP-2 inhibits G1 to S phase transition (Fig. 3), we tested the effect of BMP-2 on p21 expression in the presence and absence of EGF by immunoblot analysis. As shown in Fig. 4, EGF does not have any significant effect on p21 protein level. However, BMP-2 increases the abundance of p21 in these cells in the presence (compare lane 4 with 3) and in the absence (compare lane 2 with 1) of EGF. These data provide the first evidence that BMP-2 may inhibit MDA MB 231 cell proliferation by increasing the level of p21.

Increased association of p21 with cyclin D1 and cyclin E in the presence of BMP-2. Activation of CDKs is an important step for cell cycle progression from G1 to S phase (3). p21, by associating with cyclins D1 and E, makes them unavailable for activating CDKs and thus

TABLE 1

Flow Cytometric Analysis of MDA MB 231 Cells for G1 and S Phase Quantitation

	% of cells in G1 phase	% of cells in S phase
Control	40.86	19.40
EGF	35.51	24.15
BMP-2	45.32	12.10
EGF + BMP-2	42.93	12.87

Note. Serum-starved MDA MB 231 cells were treated with 100 ng/ml EGF in the presence and absence of 100 ng/ml BMP-2. The cells were analyzed by flow cytometric technique for quantitation of cells present in G1 and S phase.

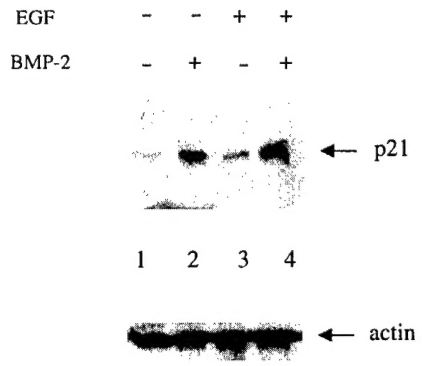

FIG. 4. Effect of BMP-2 on the cyclin kinase inhibitor p21 expression. Serum-deprived MDA MB 231 cells were incubated with EGF in the presence and absence of BMP-2. Equal amounts of cell lysates were analyzed by immunoblotting with p21 antibody and the signal was developed by ECL as described under Materials and Methods. Arrow indicates the position of p21 protein (upper panel). Lower panel shows immunoblotting of same samples with anti-actin antibody.

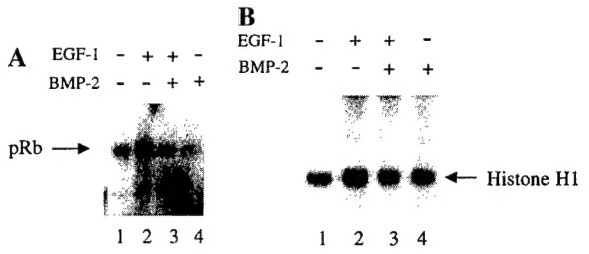

FIG. 6. Effect of BMP-2 on EGF-induced cyclin D1- and cyclin E-associated kinase activity. Serum-deprived MDA MB 231 cells were incubated with EGF in the presence or absence of BMP-2. The lysates were immunoprecipitated with cyclin D1 (A) or cyclin E (B) antibodies. The immunoprecipitates were assayed for kinase activity in the presence of [γ^{32}P]ATP using pRb (A) or histone H1 (B) as substrates. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The arrow indicates the phosphorylated pRb (A) and histone H1 (B).

inhibits cell cycle progression to S phase (7–9). Since BMP-2 increased the level of p21 (Fig. 4), we investigated whether p21 associates with cyclin D1 and cyclin E in BMP-2-treated MDA MB 231 cells in the presence or absence of EGF. p21 was immunoprecipitated from cells treated with EGF and combination of BMP-2 and EGF. The immunoprecipitates were immunoblotted either with cyclin D1 (Fig. 5A) or with cyclin E (Fig. 5B) antibody. The results show that EGF does not have any significant effect on association of p21 with these two cyclins. In contrast, incubation of cells with BMP-2 results in increased association of p21 with cyclin D1 and cyclin E in the presence (compare lanes 3 with 1 in Figs. 5A and 5B) or in the absence (compare lanes 2 with 4) of EGF. These data indicate that increased association of p21 with cyclin D1 and E may inhibit the kinase activities associated with these cyclins.

BMP-2 treatment inhibits kinase activity associated with cyclin D1 and cyclin E. Cell cycle progression depends on phosphorylation of a number of regulatory

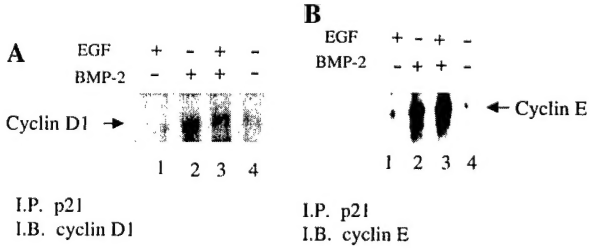

FIG. 5. Effect of BMP-2 on association of p21 with cyclin D1 and cyclin E. Equal amounts of cleared cell lysates from MDA MB 231 cells, treated with EGF in the presence or absence of BMP-2, were immunoprecipitated (I.P.) with anti-p21 antibody. The immunoprecipitates were analyzed by immunoblotting (I.B.) with anti-cyclin D1 antibody (A) and anti-cyclin E antibody (B). The arrows indicate the positions of cyclin D1 and cyclin E in A and B, respectively.

proteins by CDKs. In the G1 phase of cell cycle, cyclin D1-associated kinase is activated to initiate the cells to progress into S phase (4, 5). Since BMP-2 increases the association of cyclin kinase inhibitor p21 with cyclin D1 (Fig. 5A) and inhibits G1 to S phase progression in MDA MB 231 cells (Fig. 3), we investigated the effect of BMP-2 on cyclin D1-associated kinase activity. The lysates of MDA MB 231 cells treated with either EGF or EGF plus BMP-2 were immunoprecipitated with cyclin D1 antibody and assayed for associated kinase activity using pRb as substrate. As shown in Fig. 6A, EGF increases the pRb phosphorylation by cyclin D1-associated kinase (compare lane 2 with lane 1). BMP-2 inhibits EGF-induced pRb phosphorylation (compare lane 4 with lane 2). In mid G1 to S phase of cell cycle, cyclin E-associated kinase is activated (5, 25). Therefore, we immunoprecipitated cyclin E from lysates of MDA MB 231 cells and assayed for associated kinase activity using histone H1 as substrate. Figure 6B shows that EGF stimulates cyclin E-associated kinase activity (compare lane 2 with lane 1). However, BMP-2 inhibits EGF-induced histone H1 kinase activity (compare lane 4 with lane 2). During cell cycle progression, CDK2 remains activated in the S phase. Immunecomplex kinase assay of CDK2 immunoprecipitates showed increased CDK2 activity by EGF and BMP-2 inhibited EGF-induced CDK2 activity (data not shown). These data indicate that BMP-2 intercepts cyclin-dependent kinases to inhibit cell cycle progression of MDA MB 231 cells.

BMP-2 blocks pRb phosphorylation in MDA MB 231 cells. Activation of CDKs during mid G1 and S phase of cell cycle phosphorylate the retinoblastoma tumor suppressor protein pRb to drive the cells through cell cycle (6, 7). In MDA MB 231 cells the level of pRb phosphorylation was determined using a phospho-pRb antibody. An immunoblot analysis of lysates of cells treated with EGF alone and EGF plus BMP-2 is shown in Fig. 7. Lysates from control and

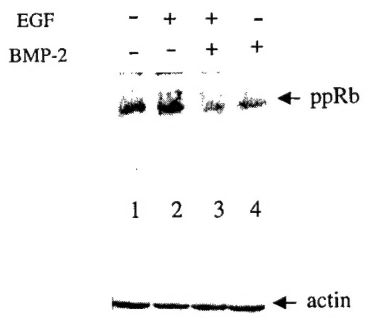

FIG. 7. Effect of BMP-2 on EGF-induced pRb phosphorylation. 50 μg cleared cell lysates of MDA MB 231 cells, treated with EGF in the presence or absence of BMP-2, were immunoblotted with an anti-phospho-pRb antibody. The arrow indicates phosphorylated pRb (ppRb).

EGF-treated cells show increased pRb phosphorylation (lanes 1 and 2). But in cells treated with BMP-2, the level of total phosphorylated pRb is significantly reduced (lane 3 and 4). One reason of increased phosphorylation in control MDA MB 231 cells may be due to their aggressive growth even in the absence of serum. These data provide the first evidence that BMP-2 inhibits pRb phosphorylation and indicate that this inhibition of pRb phosphorylation by cyclin-dependent kinases may result in attenuation of MDA MB 231 cell proliferation.

DISCUSSION

Our data in this study provide the first evidence that BMP-2 inhibits EGF-induced proliferation of MDA MB 231 cells by blocking cyclin dependent kinase activities and pRb phosphorylation. This inhibition may be due to BMP-2-induced increase in p21 cyclin kinase inhibitor level in these cells.

Breast cancer cells often metastasize to bone presumably because of a favorable growth-promoting environment provided by the bone where various growth factors are expressed in abundance (26). Our data show that one of the potent osteogenic factors, BMP-2, inhibits proliferation of MDA MB 231 cells in culture (Fig. 1). This observation was unexpected, as it was logical to predict that BMP-2, which is present in the microenvironment of bone, may not have any effect, or it may support growth of these cancer cells present in the microenvironment of bone. However, BMP-2 is present in bone matrix at a very low concentration (1–2 ng/g), whereas other growth factors such as insulin-like growth factor II (IGFII) is present at a concentration of 1500 ng/g (26, 27). Thus it is possible that the concentration of BMP-2 in the bone microenvironment may not be sufficient to inhibit the breast cancer cell growth. This may explain the requirement of a high concentration of BMP-2 to inhibit the proliferation of MDA MB 231 cells in culture (Fig. 1). Also we have shown previously that BMP-2 inhibits proliferation of primary mesangial cells at a very high concentration (19, 20). It should be noted that inhibition of prostate cancer cell proliferation is also achieved at a relatively high concentration (17).

EGF receptors in many breast cancer cells play an important role in the pathogenesis of tumor cell proliferation (24). Activation of EGFR stimulates its intrinsic tyrosine kinase activity and recruitment of cytosolic signaling proteins (28). The signals generated in the cytosol converge in the nucleus and activate cell cycle progression of breast tumor cells. EGF is a modest mitogen for MDA MB 231 breast cancer cells and we show slight induction of cell proliferation and G1 to S phase progression in these cells (Figs. 2 and 3). BMP-2 inhibited MDA MB 231 cell proliferation regardless of the presence of EGF (Figs. 1 and 2). Our results indicate that BMP-2 treatment arrests MDA cells at the G1 phase of cell cycle.

Nuclear targets of growth factor-mediated induction of cell proliferation are the cell cycle regulatory proteins (3–7). Cyclin D1 and E regulate the progression of cells in the G1 to S phase of cell cycle (5, 25). CDKs are regulated by cyclin kinase inhibitors (7, 8, 29). One of these proteins, p21, is a potent inhibitor of CDKs associated with cyclins D and E. It has been shown previously that p21 stimulates withdrawal from the cell cycle coupled to terminal differentiation (30). In breast carcinomas, increased expression of p21 was associated with relapse free survival (31). In addition to inhibition of CDKs, p21 inhibits DNA replication directly by binding to PCNA (32). In the present study we show that BMP-2 increases the level of p21 in MDA MB 231 breast tumor cells (Fig. 4). These data indicate that BMP-2-induced reduction in cell proliferation and S-phase entry may be due to the increased expression of p21 protein.

Increased cyclin D1-associated kinase activity is associated with increased proliferation of breast cancer cells (33). One of the mechanisms by which p21 blocks cell cycle progression is via interaction with cyclin D1 and cyclin E, subsequently resulting in inhibition of CDK activity (7–9). Our results show that BMP-2 increases the association of p21 with cyclin D1 and cyclin E in MDA MB 231 cells (Fig. 5) resulting in inhibition of their associated kinase activities (Fig. 6). Thus one of the mechanisms by which BMP-2 may inhibit MDA MB 231 cell proliferation is by inhibiting cyclin dependent kinases that are known to be activated in mid to late G1 and S phases of cell cycle.

During G1 phase of cell cycle the transcription factor E2F is associated with hypophosphorylated pRb. Cyclin dependent kinases phosphorylate pRb (34). pRb is also hyperphosphorylated in various breast cancer cells and tissues (35). Thus phosphorylated and inactivated pRb releases E2F transcrip-

tion factor which then activates transcription of a number of important genes necessary for cells to enter into S phase (34). In the present study we have shown that pRb is hyperphosphorylated in the presence of EGF in MDA MB 231 cells and BMP-2 causes reduction in the level of pRb phosphorylation in the presence and absence of EGF (Fig. 7). This observation provides one of the first mechanisms by which BMP-2 may inhibit MDA MB 231 breast cancer cell proliferation. Our findings may have important therapeutic implications in breast cancer treatment once the mechanism of action of BMP-2 has been characterized in more detail.

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